7,8-Dihydroneopterin-mediated protection of low density lipoprotein, but not human macrophages, from oxidative stress

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Abbreviations

3-HAA	3-Hydroxyanthranilic acid
6-PTPS	6-Pyruvol tetra-hydropterin synthase
7,8-NP	7,8-Dihydroneopterin
7,8-XP	7,8-Dihydroxanthopterin
AAPH	2,2'-azobis (2-amidinopropane) dihydrochloride
ABC1	ATP binding cassette transporter-1
$ABTS^{+}$	2,2'-Azino-di-[3-ethylbenzthaizoline sulphonate]
ACAT	Acyl-CoA: cholesterol-O-acyl-transferase
ANOVA	One-way analysis of variance
AP-1	Activator protein-1
apoB100	Apolipoprotein B100
ApoE	Apolipoprotein E
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCS	Bathocuproine disulphonic acid
BH_4	5,6,7,8-Tetrahydrobiopterin
BHT	Butylated hydroxytoluene
BPS	Bathophenanthroline disulphonic acid
BSA	Bovine serum albumin
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyltransferase
CCR2	Monocyte chemotactic protein receptor
CD40	Cluster of differentiation 40
CD40L	CD40 Ligand
cGMP	Cyclic guanosine monophosphate
CNTP	3-Carboxylato-4-nitrothiophenolate
CRP	C reactive protein
Cu^{+} or Cu(I)	Cuprous ion
Cu^{2+} or Cu(II)	Cupric ion
$CuCl_2$	Copper chloride

CuZn SOD	Copper-zinc superoxide dismutase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNPH	2,4-Dinitrophenyl hydrazine
DOPA	3,4-Dihydroxyphenylalanine
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DTNB	5,5'-Dithiobis (2-nitrobenzoic acid)
EBSS	Earle's balanced salt solution
ECD	Electrochemical detector
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
Fe^{2+} or Fe(II)	Ferrous ion
Fe^{3+} or Fe(III)	Ferric ion
$FeSO_4$	Ferrous sulphate
Fe-XO	Ferric ion-xylenol orange complex
FOX	Ferric-xylenol orange
GSH	Glutathione
GTP	Guanosine triphosphate
GuHCl	Guanidine hydrochloride
H_2O	Water
H_2SO_4	Sulphuric acid
HBSS	Hanks' balanced salt solution
HCl	Hydrochloric acid
HDL	High density lipoprotein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HETE	Hydroxyeicosatetraenoic acid
HIFCS	Heat-inactivated foetal calf serum
HIV	Human immunodeficiency virus
HMDMs	Human monocyte-derived macrophages
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HNE	4-Hydroxynonenal
HPLC	High Performance Liquid Chromatography
Hsp65	Heat shock protein 65

HTLV-1	Type 1 human T-cell leukaemia virus
ICAM-1	Intercellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
IEL	Internal elastic lamina
IFN-γ	Interferon-γ
IL-1	Interleukin-1
iNOS	Inducible nitric oxide synthase
$L^{\bullet}$	Lipid radical
LDL	Low density lipoprotein
LFA-1	Leukocyte function associated antigen-1
LO	Lipoxygenase
$LO^{\bullet}$	Lipid alkoxyl radical
LOH	Lipid hydroxide
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid hydroperoxide
LOX-1	Lectin-like oxLDL receptor-1
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRP	LDL receptor-related protein
LTR	Long terminal repeat
MANOVA	Multiple analysis of variance
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
M-CSF	Monocyte colony stimulating factor
MDA	Malondialdehyde
MHC	Major histocompatibility complex
mmLDL	Minimally modified LDL
MMP-1	Matrix metalloproteinase-1
MMP-9	Matrix metalloproteinase-9
MnSOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
N_2	Nitrogen

NAC	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium hydrogen carbonate
NBT	Nitroblue tetrazolium
NF-κβ	Nuclear factor-κβ
O_2	Oxygen
oxLDL	Oxidized LDL
PB-DOPA	Protein bound-3,4-dihydroxyphenylalanine
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Perchloric acid
PDTC	Pyrollidine dithiocarbamate
PECAM	Platelet endothelial cell adhesion molecule
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PPARγ	Peroxisome proliferator-activated receptor-γ
$PrO^{\bullet}$	Protein alkoxyl radical
PrOH	Protein hydroxide
$PrOO^{\bullet}$	Protein peroxyl radical
PrOOH	Protein hydroperoxide
PUFA or LH	Polyunsaturated fatty acid
$R^{\bullet}$	Free radical
REM	Relative electrophoretic mobility
R-N=N-R	Azo compound
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SARS	Severe acute respiratory syndrome
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SMCs	Smooth muscle cells
SOD	Superoxide dismutase

SRA	Scavenger receptor A
SR-BI	Scavenger receptor BI
SR-PSOX	Scavenger receptor that binds phosphatidylserine and oxLDL
TBA	2-Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TIMP-1	Tissue inhibitor of metalloproteinase-1
TMP	Tocopherol-mediated peroxidation
TNFα	Tumour necrosis factor-α
TPMET	Transplasma membrane electron transport
VCAM-1	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4
VLDL	Very low density lipoprotein
XO	Xylenol orange
α-MSH	α-Melanocyte-stimulating hormone
α-Toc$^{\bullet}$	α-Tocopheroxyl radical
α-TocH	α-Tocopherol

Abstract

Any lipoproteins and cells present in the inflammatory environment of atherosclerotic plaques are likely to be exposed to high levels of oxidative stress. As 7,8-dihydro-neopterin (7,8-NP) is synthesized by interferon-γ (IFN-γ)-activated macrophages, this pteridine is also thought to exist at sites of inflammation. 7,8-NP's *in vivo* role remains controversial, but numerous *in vitro* studies have identified a radical scavenging activity. The possibility of 7,8-NP protecting against oxidative damage in inflammatory environments like plaque was investigated in this thesis. Both human monocyte-derived macrophages (HMDMs) and low density lipoprotein (LDL) were used as substrates. The extent of protein hydroperoxide formation in each model, and 7,8-NP's effect on this process, were specifically studied since most previous research has focussed on lipid rather than protein peroxidation.

For the first time, neopterin (including oxidized 7,8-NP) was also directly detected by high performance liquid chromatography in the inflammatory environments of 19 pus and two atherosclerotic plaque samples. Peak concentrations even reached the low micromolar range. The positive correlation identified in the pus between neopterin and a well known antioxidant, vitamin E, further hinted at a potential antioxidant function. However, no significant association was noted between neopterin and markers of protein or lipid oxidation.

Exposure of HMDMs to the AAPH peroxyl radical generator resulted in significant quantities of lipid hydroperoxides but not protein hydroperoxides, as detected by the FOX assays. This is likely due to the large accumulation of polyunsaturated fatty acid-rich lipid in the primary HMDMs during differentiation in 10% human serum and is of relevance to atherosclerotic plaque, where macrophages also become lipid-loaded. The addition of up to 200μM 7,8-NP failed to prevent AAPH-induced lipid peroxidation and was also unable to inhibit a loss of cellular thiols or viability. This lack of effect suggests the damaging peroxyl radicals are not being scavenged by 7,8-NP. The high lipid content of HMDM cells appears to cause the AAPH and/or 7,8-NP to localize to a cellular site, where they are unable to interact.

Macrophage-mediated oxidation of LDL in iron(II)-supplemented Hams F10 was associated with the formation of 30-40 moles of protein hydroperoxides per mole of LDL. The close parallel between protein and lipid peroxidation supports the theory that lipid-derived radicals are involved in protein hydroperoxide formation on LDL and

indicates that protein hydroperoxides are an early product of LDL oxidation. Their detection during exposure of LDL to both the THP-1 macrophage cell line and primary HMDM cells confirms that protein hydroperoxides are also a normal consequence of macrophage-mediated LDL oxidation.

Incubation of LDL with micromolar 7,8-NP prevented macrophage-mediated protein hydroperoxide formation in a concentration-dependent manner. Lipid oxidation and vitamin E loss were similarly inhibited by 7,8-NP during the cell-mediated attack of LDL. Kinetic analysis revealed protection due to extension of the lag phase, with 7,8-NP depletion and initiation of the propagation phase coinciding. This supports a radical scavenging activity for 7,8-NP, resulting in protection of the entire LDL particle. By contrast, the release of nanomolar quantities of 7,8-NP by IFN-γ-stimulated THP-1 macrophages failed to prevent LDL oxidation. HMDMs activated by IFN-γ did significantly inhibit LDL oxidation, including protein hydroperoxide formation, for up to 48 hours but this antioxidant effect was not due to the *de novo* synthesis of 7,8-NP.

These results indicate that both the prevalence of protein hydroperoxides, and the ability of 7,8-NP to act as an antioxidant, depend on the system under investigation. Neopterin exists in inflammatory environments but, considering the lack of protection against AAPH-mediated HMDM oxidation and the 7,8-NP concentration required to inhibit macrophage-mediated LDL oxidation, strong evidence for an antioxidant activity of 7,8-NP in atherosclerotic plaque is currently lacking.

1 Introduction

1.1 Overview

As atherosclerotic plaques represent sites of chronic inflammation, the lipoproteins and cells present in such environments are likely to be exposed to high levels of oxidative stress. Oxidative damage to both cells and lipoproteins has been detected in atherosclerotic lesions (Yla-Herttuala *et al.*, 1989; Martinet *et al.*, 2001). In an attempt to protect themselves from this damage, macrophages have evolved a range of cellular responses including, but not limited to, antioxidant production. 7,8-Dihydroneopterin (7,8-NP) may be one of these antioxidants because, although its *in vivo* role remains controversial, numerous *in vitro* studies have provided evidence of antioxidant activity at micromolar concentrations (for review, see Oettl & Reibnegger, 2002). Interferon-γ (IFN-γ) stimulates human macrophages to synthesize 7,8-NP and, as this cytokine has been detected in atherosclerotic plaque (Geng *et al.*, 1995; Frostegard *et al.*, 1999), the potential exists for 7,8-NP to be present in these lesions as well. Consequently, 7,8-NP may be able to inhibit oxidative damage to both macrophages and LDL in the inflammatory environment of the plaque.

This hypothesis will be investigated in two separate studies. The first will model oxidative stress to human monocyte-derived macrophages (HMDMs) by exposure to a peroxyl radical generator. The second will promote cell-mediated LDL oxidation using both the THP-1 monocyte cell line and the more physiologically relevant HMDMs. 7,8-NP's presence in the inflammatory environments of pus and atherosclerotic plaque will also, ultimately, be confirmed.

1.2 Atherosclerosis

It is now widely recognized that atherosclerosis is not merely a problem of blocked arteries but is, in fact, a multifactorial disease. Oxidative stress and inflammation represent particularly critical components of the pathological process. Risk factors therefore range from hyperlipidemia, obesity, lack of exercise and hypertension to smoking, diabetes, hyperhomocysteinemia and even infection (Scott, 2004; Wu & Wu, 2006).

Atherosclerosis is also a slowly developing disease, spanning many decades in humans. Some of the earliest signs of atherosclerosis, termed fatty streaks, have been detected in adolescents (McGill *et al.*, 2000) but the clinical events are predominantly observed only in people over 50 years of age (Milne *et al.*, 2003).

1.2.1 Development of the Plaque

1.2.1.1 Lesion Initiation

Atherosclerosis begins as sub-clinical fatty streaks within the intima of arteries. Arterial branch points are particularly susceptible sites because the turbulent blood flow in these regions alters the release of various endothelium-derived cytokines. This creates a more atherogenic profile by disrupting endothelium-dependent vasodilation (McLenachan *et al.*, 1991) and increasing the adherence of cells to the endothelium (Ross, 1993). Turbulent blood flow is also likely to affect endothelial cell morphology (Lusis, 2000) and cause chronic injury to cells (Jang *et al.*, 1993). The former provides one explanation for the increased permeability of LDL at these arterial branch sites (Lusis, 2000) and the latter would lead to chronic inflammation, a feature commonly associated with atherosclerosis (Jang *et al.*, 1993).

Lipid-filled macrophages (foam cells) are the predominant component of fatty streaks, though small quantities of T lymphocytes have also been detected (Stary *et al.*, 1994). Despite many detailed analyses of fatty streak composition, controversy still surrounds the exact sequence of events during the early stages of formation. Some suggest a response to injury hypothesis, involving monocyte recruitment to the injured endothelium and subsequent migration into the intima (Jang *et al.*, 1993). Lipoprotein entry into the intima would occur soon afterwards. Alternatively, the lipoproteins may enter first and initially become modified in the absence of any monocytes/macrophages. The appearance of these cells at a later stage would then promote the further progression of the atherogenic process. In support of the latter hypothesis, oxidized LDL (oxLDL) has been immunohistochemically identified in foetal aortae prior to the detection of any monocytes (Napoli *et al.*, 1997). A similar result has been observed in rats following injection with native human LDL (Calara *et al.*, 1998).

Whatever the initial sequence of events, monocyte recruitment is clearly an important step in atherogenesis (Figure 1.1). Macrophages are one of the most prominent cell types in plaque (Stary *et al.*, 1994; Stary *et al.*, 1995) and mice

genetically engineered to lack large numbers of macrophages develop atherosclerosis to a significantly less extent than control mice (Smith *et al.*, 1995; Qiao *et al.*, 1997). Entry into the arterial intima initially requires weak and rolling interactions between L-selectin on the monocytes and endothelial-associated P- and E-selectin (Dong *et al.*, 1998). Stronger interactions between the monocyte and endothelial cells are subsequently established and include recognition between the monocytic integrin very late antigen (VLA-4) and endothelial vascular cell adhesion molecule (VCAM-1). Interactions between the monocytic leukocyte function associated antigen (LFA-1) and endothelial intercellular adhesion molecule (ICAM-1) have also been reported (Lusis, 2000). Firm attachment to endothelial cells is followed by migration of the monocytes into the intima through endothelial tight junctions. Platelet endothelial cell adhesion molecule (PECAM) is a prime candidate for mediating the monocyte-endothelial interactions required for this movement (Scott, 2004). Monocyte migration is further aided by chemotactic factors. Monocyte chemotactic protein (MCP-1) and its corresponding monocyte receptor, CCR2, appear to be particularly important because atherosclerotic lesions are reduced in MCP-$1^{-/-}$ (Gu *et al.*, 1998; Gosling *et al.*, 1999) or $CCR2^{-/-}$ (Boring *et al.*, 1998) mice. Once in the intima, monocytes are ultimately induced to differentiate into macrophages by exposure to cytokines like monocyte colony stimulating factor (M-CSF) and IFN-γ (Glass & Witztum, 2001).

T lymphocyte recruitment is also critical to atherosclerosis, with the $RAG^{-/-}$ mice deficient in this cell type exhibiting retarded lesion development compared to controls (Song *et al.*, 2001). These differences diminish over time, placing particular emphasis on the role of T cells during early stages of the disease.

LDL modification represents another important feature of atherogenesis. This modification is generally described as being oxidative in nature and it causes macrophages to endocytose the modified LDL via their scavenger receptors. Although scavenger receptor AI/AII (SRA) and CD36 are considered to be the most significant receptors (Suzuki *et al.*, 1997; Febbraio *et al.*, 2000), several others have been identified in recent years. These include CD68/macrosialin (van der Kooij *et al.*, 1997), scavenger receptor BI (SR-BI) (Gillotte-Taylor *et al.*, 2001), lectin-like oxLDL receptor (LOX-1) (Yoshida *et al.*, 1998) and scavenger receptor that binds phosphatidylserine and oxLDL (SR-PSOX) (Shimaoka *et al.*, 2000). Irrespective of the exact scavenger receptor, the unregulated nature of this oxLDL uptake appears to generate lipid-laden macrophage foam cells. OxLDL, itself (section 1.2.3), and mechanical stresses designed to mimic

the conditions of turbulent blood flow or hypertension (Sakamoto *et al.*, 2001), are just two factors known to increase SRA and CD36 expression in human monocytes/ macrophages. Such mechanisms are likely to contribute to the further enhancement of foam cell formation, as will scavenger receptor-independent pathways of oxLDL uptake. In particular, aggregated oxLDL and antibody-tagged oxLDL can be taken up via non-specific phagocytosis (Steinbrecher & Lougheed, 1992) and the Fc-γ receptor (Morganelli *et al.*, 2000), respectively. Conversely, homeostatic responses provide a limited means of reducing foam cell formation by promoting the efflux of cholesterol from macrophages to high density lipoprotein (HDL) (Glass & Witztum, 2001).

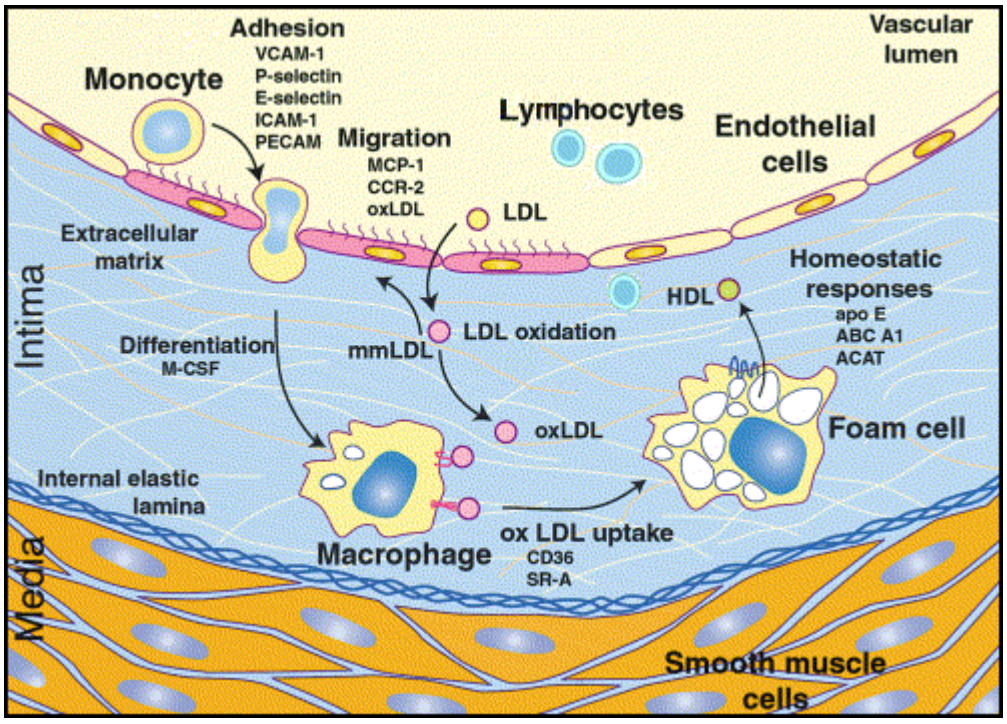


Figure 1.1 Development of the fatty streak.
Adapted from Glass & Witzum (2001) and Scott (2004).

1.2.1.2 Lesion Progression

The next stage of plaque development is characterized by the migration of smooth muscle cells (SMCs) from the vascular media into the intima (Figure 1.2). In contrast to the contractile phenotype associated with SMCs below the internal elastic lamina (IEL), the newly migrated cells possess a synthetic phenotype (Ross, 1993). This phenotype permits the expression of various growth regulatory cytokines and receptors, which, in combination with the cytokines and growth factors released from macrophages and T lymphocytes, results in SMC proliferation. It also permits the release of extracellular

matrix material, leading to the development of a fibrous cap over the plaque. Components of this extracellular matrix are composed primarily of type I collagen, but proteoglycans, elastin, glycoproteins, fibrin and other forms of collagen are also present (Jang *et al.*, 1993; Stary *et al.*, 1995).

The plaque may also become more complex, hardening the fibrous cap further via calcification of the fibrous material. Apatite is the major mineral in calcified lesions and tends to be found in the extracellular matrix associated with elastin and vesicles derived from dead cells (Stary *et al.*, 1995). As a result, both these plaque components are suggested to serve as critical sites for calcification.

Foam cells continue to form during this stage of atherogenesis and, in addition to macrophages, some SMCs also develop a foam-like appearance (Figure 1.3). This has been suggested to occur, at least in part, by scavenging lipid released upon the death of the macrophage foam cells (Berliner *et al.*, 1995). Ultimately, however, the predominant role for lipid and ceroid released from dead macrophages arises from their contribution to the development of an acellular necrotic core (Ball *et al.*, 1995). Macrophages that participate in the formation of the necrotic core do not all die by necrosis. Apoptosis is just as plausible and, in fact, apoptotic nuclei have been directly detected within this core (Bjorkerud & Bjorkerud, 1996; Hegyi *et al.*, 1996). The term necrotic core is, thus, rather misleading and many papers now refer to it as an acellular lipid core.

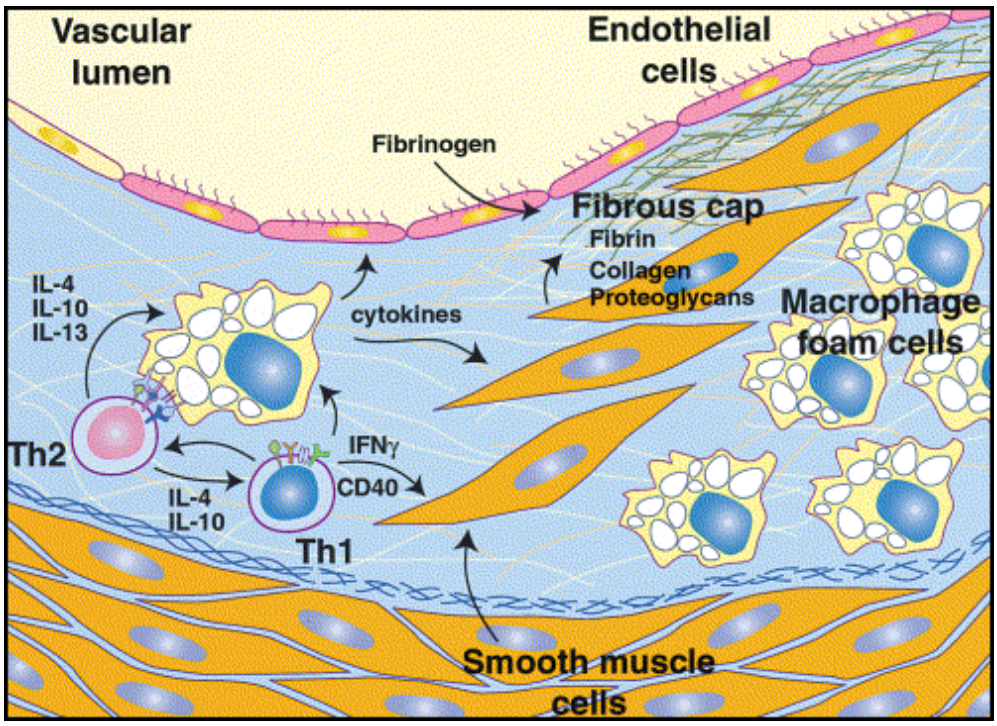


Figure 1.2 Lesion progression from a fatty streak to a fibrous plaque.
Adapted from Glass & Witzum (2001).

1.2.1.3 Plaque Rupture and Thrombosis

The continued monocyte recruitment, foam cell formation, necrotic core expansion, SMC migration and proliferation, and extracellular matrix deposition, may eventually cause occlusion of the artery lumen. However, stenosis is generally a poor indicator of risk of an acute cardiovascular event (Libby, 1995). Heart attacks are, instead, predominantly caused by rupture of the fibrous cap and subsequent thrombosis. The likelihood of this occurring obviously depends on the stability of the fibrous cap and not on the extent of stenosis. A stable plaque is characterized by a thick fibrous cap, large amounts of SMCs and low levels of inflammatory cells (Scott, 2004). By contrast, unstable plaques are typified by a thin fibrous cap, with large quantities of lipid-filled macrophages, T lymphocytes and debris from dead cells. More specifically, plaque rupture is often localized to shoulder regions of the atherosclerotic lesion where macrophage cells are abundant (Bjorkerud & Bjorkerud, 1996).

As these observations suggest, inflammatory cells play a particularly critical role in destabilizing the fibrous cap (Figure 1.3). Activated T lymphocytes secrete IFN-γ (Libby & Hansson, 1991), which is directly responsible for many of the destabilizing effects (section 1.2.4.1). This cytokine also activates macrophages and thus, in combination with other stimuli, induces macrophages to secrete matrix metalloproteinase-1 (MMP-1) (Anderson *et al.*, 2002). MMPs degrade extracellular matrix material, thereby thinning the fibrous cap and making it more susceptible to rupture. Considering that IFN-γ (Geng *et al.*, 1995; Frostegard *et al.*, 1999) and active MMPs (Galis *et al.*, 1994) have both been detected at sites of plaque rupture, the likelihood of such reactions occurring in atherosclerotic lesions is certainly high.

Plaque stability may be further disrupted by the death of macrophages and SMCs, particularly if death occurs near the surface of the atherosclerotic lesion. It is therefore noteworthy that apoptotic macrophages and SMCs have been identified in the shoulder region and fibrous cap of plaques (Bjorkerud & Bjorkerud, 1996; Kolodgie *et al.*, 2000). Moreover, the pro-apoptotic Fas receptor appears to be expressed on up to 66% of all cells in the fibrous cap (Haunstetter & Izumo, 1998).

Microvessel formation within atherosclerotic lesions also affects plaque stability. Such vessels are commonly detected in atherosclerotic lesions and are prone to rupture (Libby, 1995). These intra-plaque haemorrhages are often absorbed into the lesion, thereby contributing both to plaque growth and plaque instability (Kolodgie *et al.*, 2003; Takaya *et al.*, 2005).

Tissue factor plays a central role in initiating coagulation and is yet another abundant component of atherosclerotic plaques (Wilcox *et al.*, 1989). As a result, once the thinned fibrous cap ruptures, blood will be exposed not only to the thrombogenic necrotic core but also to this pro-coagulant tissue factor (Dickson & Gotlieb, 2003). In combination with platelets, these interactions quickly lead to the formation of a thrombus. Like intra-plaque haemorrhages, this thrombus is often absorbed into the existing, but now larger, plaque (Davies, 1996b). However, once a certain size is exceeded, the thrombus can block arteries, resulting in events such as coronary infarcts (heart attacks), cerebral infarcts (stroke) and death (Jang *et al.*, 1993).

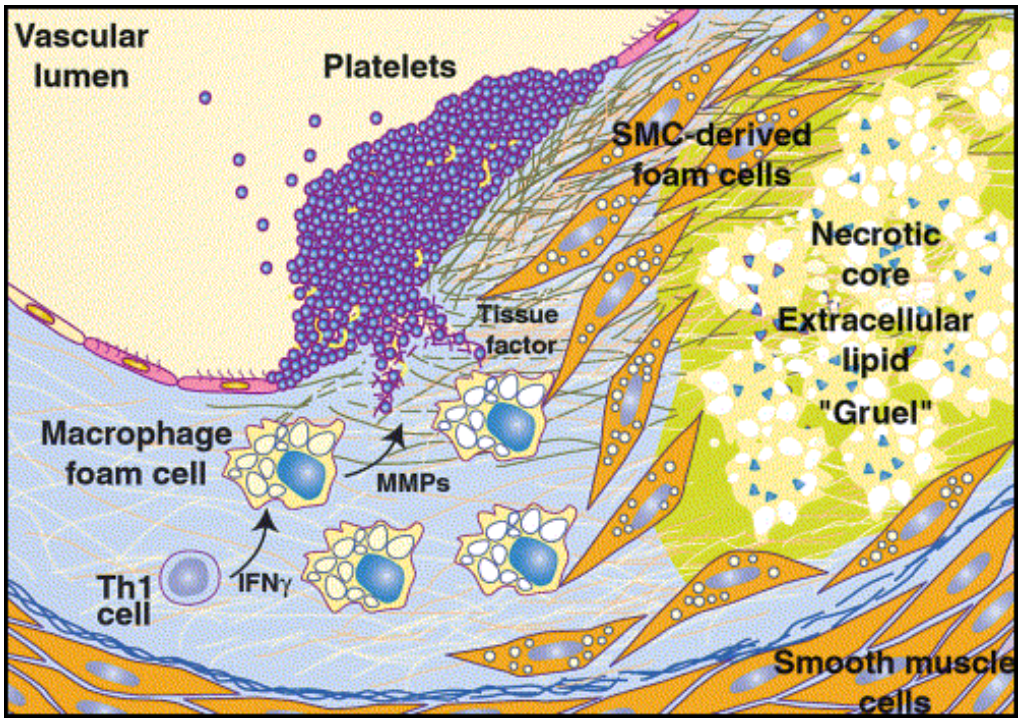


Figure 1.3 Advanced plaque, with rupture and thrombosis.
Adapted from Glass & Witzum (2001).

1.2.2 Low Density Lipoprotein

It has long been known that elevated plasma LDL levels are associated with an increased risk of atherosclerosis (Steinberg *et al.*, 1989). Nevertheless, it is also understood that native LDL is incapable of promoting foam cell formation and must first be modified to a form recognized by the macrophage scavenger receptors for unregulated uptake. Acetylated LDL was one of the first such modifications identified (Brown *et al.*, 1979) but is not physiologically relevant because it does not exist *in vivo*

(Steinberg *et al.*, 1989). By contrast, oxLDL has been detected *in vivo* and is also recognized by scavenger receptors.

Support for oxLDL's existence arises from several lines of evidence, including the finding that copper-oxidized LDL induces a response in T lymphocytes isolated from atherosclerotic plaque but not the corresponding peripheral blood (Stemme *et al.*, 1995). Autoantibodies (Salonen *et al.*, 1992; Shaw *et al.*, 2001) and immunoglobulins (Yla-Herttuala *et al.*, 1994) reactive towards copper-oxidized LDL have also been isolated from human serum and plaque, respectively. Furthermore, human atherosclerotic lesions but not healthy arteries are clearly stained by antibodies designed against epitopes of oxLDL (Yla-Herttuala *et al.*, 1991; Itabe *et al.*, 1994). Unfortunately, such antibodies are not 100% specific for oxLDL (Chisolm & Chai, 2000). Thus, the direct detection of oxidation markers on plaque-derived lipoproteins provides more compelling evidence for the existence of oxLDL (Yla-Herttuala *et al.*, 1989; Niu *et al.*, 1999). It is also noteworthy that positive correlations exist between the severity of coronary atherosclerosis and the concentration of the cholesteryl ester hydro(pero)xide oxidation marker (Upston *et al.*, 2002) or the susceptibility of LDL to *in vitro* oxidation (Regnstrom *et al.*, 1992). Likewise, plasma lipid hydroperoxides (Kovacs *et al.*, 1997) and plasma LDL baseline diene conjugation (Ahotupa & Asankari, 1999) tend to be elevated in patients with coronary artery disease.

1.2.2.1 LDL Composition

LDL has evolved to transport cholesterol to peripheral tissues in the body and its composition reflects this function. It is characterized by a core of triglycerides and cholesteryl esters, surrounded by a phospholipid monolayer containing free cholesterol and the apolipoprotein B100 (apoB100) protein (Esterbauer *et al.*, 1992).

Interactions between this 4536 amino acid protein and the LDL receptor are critical for the normal, regulated receptor-mediated endocytosis of LDL by target cells (Brown & Goldstein, 1986). However, apoB100 also contributes to atherogenesis. Alterations to the apoB100 result in a loss of LDL receptor recognition but a gain of scavenger receptor recognition. This switch was found to be particularly dependent on the modification of apoB100 lysine residues rather than merely requiring a change in the net negative charge of the lipoprotein (Haberland *et al.*, 1984). ApoB100 modification may occur via direct attack of susceptible amino acid residues or, indirectly, via

derivatization with the lipid aldehydes produced during lipid hydroperoxide decomposition (Haberland *et al.*, 1984; Jurgens *et al.*, 1986; Steinbrecher, 1987).

ApoB100's interaction with proteoglycans further contributes to atherogenesis because it promotes retention of LDL in the intima and provides time for exposure to oxidative attack. A single point mutation to the histidine- and lysine-rich heparin binding domain of LDL produces a particle still recognizable by the LDL receptor but no longer able to interact with the negatively charged regions of proteoglycans (Boren *et al.*, 1998). Mice expressing this mutated apoB100 were found to develop significantly less atherosclerosis than controls, even when fed a high cholesterol diet (Skalen *et al.*, 2002).

Lipids are abundant in LDL, with an estimated 2,200 cholesterol/cholesteryl ester molecules and 2,600 fatty acids (Esterbauer *et al.*, 1995). As half these fatty acids are polyunsaturated, with a predominance of linoleic acid (Esterbauer *et al.*, 1995), the LDL particle is highly susceptible to free radical attack (Parthasarathy *et al.*, 1990b). Consequently, LDL also contains a variety of antioxidants for protection, most notably an average of six α-tocopherol molecules per lipoprotein (Esterbauer *et al.*, 1992). The remaining antioxidants are each reported to be present at less than one mole per mole of LDL and include γ-tocopherol, carotenoids, retinoids and ubiquinol-10 (Esterbauer *et al.*, 1992).

α-Tocopherol is the most effective form of vitamin E (Brigelius-Flohe & Traber, 1999) and, during the lag phase of LDL oxidation, is generally the first antioxidant to be consumed while β-carotene is the last (Esterbauer *et al.*, 1991). Traditionally, α-tocopherol (α-TocH) has been considered a chain breaking antioxidant that donates hydrogen to reactive lipid peroxyl ($LOO^{\bullet}$) radicals (reaction 1) (Burton & Ingold, 1986). Under conditions of suitably high radical flux, the resulting α-tocopheroxyl radical ($\alpha\text{-Toc}^{\bullet}$) can then neutralize a second lipid peroxyl radical (reaction 2). When the radical flux is extremely low, however, α-tocopherol exhibits pro-oxidant rather than antioxidant activities (Bowry *et al.*, 1992; Neuzil *et al.*, 1997). This occurs despite α-tocopheroxyl radicals being considerably less reactive than lipid peroxyl radicals because, in the absence of any other substrate, α-tocopheroxyl radicals will ultimately extract hydrogen from lipid (Upston *et al.*, 1999). While the abstraction regenerates α-tocopherol, it also creates a lipid radical ($L^{\bullet}$) (reaction 3) that can participate in the lipid peroxidation chain reaction.

(1) α-TocH + $LOO^{\bullet}$ → α-$Toc^{\bullet}$ + LOOH

(2) α-$Toc^{\bullet}$ + $LOO^{\bullet}$ → Non-radical product + LOOH

(3) α-$Toc^{\bullet}$ + LH → α-TocH + $L^{\bullet}$

This tocopherol-mediated peroxidation (TMP) can be prevented by co-antioxidants that neutralize the α-tocopheroxyl radical and transfer the newly created, damaging radical into the aqueous phase (Bowry *et al.*, 1995; Thomas *et al.*, 1996a; Upston *et al.*, 1999). Examples include endogenous co-antioxidants like ascorbate, ubiquinol-10 and bilirubin. The detection of vitamin E and several co-antioxidants in all stages of atherosclerotic lesions (Suarna *et al.*, 1995; Upston *et al.*, 2002) has therefore raised questions about the *in vivo* relevance of TMP. Nevertheless, it remains possible that microenvironments deficient in co-antioxidants may exist and that TMP may occur in these regions. It is also suggested that, if LDL is in close proximity to free copper, α-tocopherol may act as a pro-oxidant by reducing this transition metal to its redox active state (Yoshida *et al.*, 1994).

The importance of co-antioxidants, and their ability to act synergistically (Sato *et al.*, 1990), provides one explanation for the relatively consistent protection observed against cardiovascular disease during epidemiological studies of dietary vitamin E. This is because an increased dietary intake of vitamin E is also suggested to represent an increased intake of dietary co-antioxidants (Stocker, 1999). By extension, the contradictory results obtained from vitamin E supplementation trials in humans may be caused by a lack of co-supplementation with appropriate co-antioxidants (Stocker, 1999; Upston *et al.*, 1999; Heinecke, 2001). Additional suggestions for the inconsistency observed in human antioxidant supplementation trials range from the generally advanced stage of atherosclerosis being investigated (Hazell & Stocker, 1997; Pryor, 2000; Steinberg & Witztum, 2002; Kaliora *et al.*, 2006) to the use of inappropriate antioxidants, antioxidant doses, sample populations and trial lengths. Thus, a lack of efficacy in these studies should not necessarily be viewed as a negation of the oxidative theory of atherosclerosis.

1.2.2.2 Kinetics of LDL Oxidation

There are three characteristic phases of LDL oxidation and the first, the lag phase, represents a period of antioxidant consumption (Esterbauer *et al.*, 1992). Once the antioxidants are consumed, lipid peroxidation commences and rapidly leads to a

self-propagating chain reaction that greatly amplifies the initial damage. More specifically, the free radical ($R^{\bullet}$)-mediated abstraction of a hydrogen atom from a polyunsaturated fatty acid (PUFA; LH) (reaction 4) generates a lipid radical that subsequently reacts rapidly with molecular oxygen (reaction 5) (Porter *et al.*, 1995). The resulting peroxyl radical can then abstract an atom of hydrogen from a neighbouring lipid, producing a lipid hydroperoxide and a new lipid radical (reaction 6) that may participate in reaction 4. The length of the chain reaction depends on many factors (Esterbauer *et al.*, 1993) but will, ultimately, be terminated by reaction with antioxidants or other radicals (reactions 1 & 7).

(4) $LH + R^{\bullet} \rightarrow L^{\bullet} + RH$

(5) $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

(6) $LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$

(7) $LOO^{\bullet} + R^{\bullet}/LOO^{\bullet} \rightarrow LOOR/LOOOOL \rightarrow$ Other non-radical products

PUFAs are not the only targets of attack during the propagation phase. Time course studies have confirmed that the apoB100 of LDL is also subject to oxidative modification (Giessauf *et al.*, 1995; Knott *et al.*, 2002; Gieseg *et al.*, 2003). Likewise, the cholesterol associated with LDL is oxidized predominantly to oxysterols at the seven position. Analysis of plaque material and time course investigations have indicated that hydroperoxycholesterol is the principal oxysterol early in atherosclerosis, while 7-ketocholesterol and 7-hydroxycholesterol are later stage products (Brown *et al.*, 1997; Jessup & Kritharides, 2000; Upston *et al.*, 2002). Enzymatically generated 26/27-hydroxycholesterol has also been detected, particularly in more advanced lesions (Carpenter *et al.*, 1995; Jessup & Kritharides, 2000; Upston *et al.*, 2002).

Lipid hydroperoxides continuously break down during LDL oxidation but the final decomposition phase begins only once these reactions predominate (Esterbauer *et al.*, 1992). For copper-mediated LDL oxidation, this generally occurs once 70-80% of PUFAs have been oxidized (Esterbauer *et al.*, 1992). The end products of decomposition include hydrocarbon gases, epoxides, alcohols and especially aldehydes (Esterbauer *et al.*, 1995). This decomposition can occur spontaneously but is accelerated in the presence of transition metals, resulting in the initial formation of lipid peroxyl and lipid alkoxyl ($LO^{\bullet}$) radicals (Cheeseman & Slater, 1993). The thermodynamically unfavourable nature of reaction 9 ensures that it proceeds very slowly relative to reaction 8 (Burkitt, 2001). Whichever radical is generated, decomposition products are

ultimately formed and lipid peroxidation is further propagated via the radical-mediated abstraction of a PUFA hydrogen atom (reaction 10). Some of the aldehydes produced during this phase are cytotoxic (section 1.2.3) and may also conjugate to apoB100 (section 1.2.2.1). Thus, LDL only becomes sufficiently modified for recognition and uptake by macrophage scavenger receptors at this late stage of oxidation (Jessup *et al.*, 1990).

(8) $LOOH + Fe^{2+}/Cu^{+} \rightarrow LO^{\bullet} + Fe^{3+}/Cu^{2+} + OH^{-}$

(9) $LOOH + Fe^{3+}/Cu^{2+} \rightarrow LOO^{\bullet} + Fe^{2+}/Cu^{+} + H^{+}$

(10) $LO^{\bullet}/LOO^{\bullet} + LH \rightarrow LOH/LOOH + L^{\bullet}$

Protein hydroperoxides will be discussed in greater detail in chapter 5 but it is worth noting now that this moiety is generated (reactions 4-6) and decomposed (reactions 8-10) by mechanisms similar to the ones described for lipid hydroperoxides. As a consequence, protein hydroperoxide formation may also participate in peroxidation chain reactions (Neuzil *et al.*, 1993) while the peroxyl and alkoxyl radicals produced during decomposition have the potential to contribute to additional oxidative damage (Davies *et al.*, 1995; Gebicki *et al.*, 1995). The occurrence of protein hydroperoxides in atherosclerosis is supported by the detection of valine hydroperoxide and leucine hydroperoxide break-down products in plaque samples (Fu *et al.*, 1998).

1.2.2.3 Location of LDL Oxidation

Although the general sequence of events during LDL oxidation is rapidly being elucidated, the actual location of oxidation remains unclear. Plasma contains large quantities of antioxidants (Halliwell & Gutteridge, 1990) and as little as 5-6% human or foetal calf serum has been shown to inhibit macrophage-mediated LDL oxidation *in vitro* (Leake & Rankin, 1990; Dabbagh & Frei, 1995). Consequently, it has been hypothesized that LDL oxidation occurs predominantly in the arterial intima rather than the plasma. However, macrophage-mediated oxidation is also inhibited by the interstitial fluid that is expected to bathe the artery wall (Dabbagh & Frei, 1995). Moreover, antioxidants have been directly detected in atherosclerotic plaques (Suarna *et al.*, 1995; Upston *et al.*, 2002) and vitamin E even appears to be associated with the oxidized lipoproteins in these lesions (Niu *et al.*, 1999). As a result, TMP and other oxidative mechanisms in the intima may be restricted to microenvironments low in

antioxidants and interstitial fluid. The creation of such an environment is plausible, particularly in close proximity to cells, because cellular secretions may alter the pH as well as oxygen and antioxidant availability of the immediate surroundings (Garner & Jessup, 1996).

1.2.2.4 Mechanisms of LDL Oxidation

While controversy also surrounds the initiating mechanism of LDL oxidation *in vivo*, cells in the atherosclerotic plaque are postulated to play a critical role. Smooth muscle cells (Heinecke *et al.*, 1984; Muller *et al.*, 1998), endothelial cells (Sparrow & Olszewski, 1993; Muller *et al.*, 1998), macrophages (Carpenter *et al.*, 1994; Muller *et al.*, 1998), monocytes (Folcik *et al.*, 1997) and lymphocytes (Lamb *et al.*, 1992) have all been shown to promote LDL oxidation *in vitro*. Macrophages may be of particular relevance, both because of their ability to produce a range of oxidizing species and because of their abundance in the plaque relative to other cell types, especially during early stages of lesion development (Chisolm & Chai, 2000).

i) Transition Metals in LDL Oxidation

Numerous theories have been proposed to explain the underlying mechanism of cell-mediated LDL oxidation and most include a requirement for transition metals. Copper is a particularly potent mediator of LDL oxidation due to its ability to bind histidine residues on the apoB100 (Wagner & Heinecke, 1997; Roland *et al.*, 2001). Such close proximity permits both LDL-mediated reduction of copper to its redox active form and site-specific damage to the LDL. The former may be mediated by the α-tocopherol, lipid and/or protein components of LDL (Yoshida *et al.*, 1994; Wagner & Heinecke, 1997; Batthyany *et al.*, 2000) while the latter involves Fenton reactions (Lynch & Frei, 1993; Burkitt, 2001). Ultimately, however, the most important role for all transition metals may be propagation rather than site-specific initiation of the oxidative damage. The suggestion arises because metals decompose pre-existing lipid hydroperoxides to reactive alkoxyl/peroxyl radicals (reactions 8 & 9) and this process appears to be critical for metal-dependent lipid peroxidation (Thomas & Jackson, 1991; Pinchuk *et al.*, 1998; Tang *et al.*, 2000). Pre-existing lipid hydroperoxides may be derived from dietary lipids (Wilson *et al.*, 2002), generated by autoxidation of α-tocopherol (Burkitt, 2001) or

transferred from peroxidized cell membrane lipid to the LDL (Parthasarathy *et al.*, 1990a).

When present in sufficient quantities, transition metals can oxidize LDL under cell free conditions (Parthasarathy *et al.*, 1990a; Kritharides *et al.*, 1995a). Cells accelerate the oxidative process by continually reducing the metals back to their redox active forms, either via the action of the transplasma membrane electron transport (TPMET) system or via the release of reducing agents such as superoxide and thiols. Glucose, likewise, represents a reducing agent of potential *in vivo* relevance (Mowri *et al.*, 2000; Leoni *et al.*, 2002).

Superoxide lacks the reactivity to induce lipid peroxidation in the absence of additional reagents (Bedwell & Jessup, 1987; Chisolm *et al.*, 1999) but may do so more indirectly via transition metal reduction or participation in Fenton reactions. Despite this potential, THP-1 monocytes appear to reduce copper ions by a superoxide-independent mechanism (Wood & Graham, 1999) and clear evidence for the contribution of superoxide towards LDL oxidation is currently lacking (Jessup & Dean, 1993; Garner *et al.*, 1994; Wilkins & Leake, 1994). Studies of atherosclerosis in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-deficient mice are similarly inconclusive. While Barry-Lane *et al.* (2001) observed a reduced lesion size compared to control subjects in this model, Kirk *et al.* (2000) detected no such difference. Nevertheless, it should be noted that the former study used mice lacking the gp47phox subunit of NADPH oxidase but the latter study used gp91phox knockouts. There is some suggestion that a gp91phox deficiency may not alter NADPH oxidases in vascular cells due to the existence of compensatory homologues (Griendling & Harrison, 2001). Furthermore, although NADPH oxidase represents a major pathway for superoxide production in monocytes/macrophages, it remains possible that alternative pathways may influence atherosclerotic development (Kirk *et al.*, 2000). In an attempt to more broadly address the question of superoxide in atherosclerosis, mice were engineered to overexpress copper-zinc superoxide dismutase (CuZn SOD) (Tribble *et al.*, 1997). These transgenic mice were just as prone to disease as controls but, because the relevance of intracellular CuZn SOD in atherosclerotic development is ambiguous, the data from this study also remains inconclusive.

The failure to identify a clear link between superoxide and atherosclerosis further suggests that the superoxide derived from thiol or glucose autoxidation may not play a critical role in LDL oxidation *in vivo*. Indeed, thiol-mediated LDL oxidation has been

correlated with susceptibility to autoxidation rather than superoxide generation (Wood & Graham, 1995). Additional products of thiol autoxidation include thiyl and thiyl-peroxyl radicals, both of which are capable of initiating lipid peroxidation (Halliwell & Gutteridge, 1999). The most critical outcome of thiol autoxidation, however, may be the reduction of transition metals back to their redox active forms. Several studies have clearly demonstrated enhanced endothelial- and macrophage-mediated oxidation of LDL upon addition of extracellular cystine to transition metal-containing media (Sparrow & Olszewski, 1993; Graham *et al.*, 1994; Garner *et al.*, 1997b). Cells are suggested to promote LDL oxidation under these conditions by continual uptake of the L-cystine, with subsequent intracellular reduction and export. This ensures that the resulting cysteine is once more extracellularly available for autoxidation and concomitant reduction of transition metals. Wood & Graham (1999) provided support for this theory by confirming that increased cystine uptake and thiol export in THP-1 macrophages correlates positively with rates of transition metal reduction.

The Wood & Graham (1999) study further indicated that a thiol-independent reduction of transition metals was also occurring, possibly via TPMET. This TPMET system is ubiquitous to all mammalian cells and functions by transferring electrons from intra-cellular nicotinamide adenine dinucleotide (NADH) to extracellular substrates. Cellular activities, ranging from cell growth and differentiation to iron uptake and defence, are all suggested to be modulated by TPMET (Ly & Lawen, 2003). Furthermore, it is suggested to participate in atherosclerosis by reducing extracellular transition metals to their redox active forms (Garner *et al.*, 1997b; Baoutina *et al.*, 2000). One study has even identified a direct correlation between TPMET activity and the extent of cell-mediated LDL oxidation (Baoutina *et al.*, 2001b).

Despite all this *in vitro* evidence for metal-mediated LDL oxidation, the role of transition metals *in vivo* remains controversial. Admittedly, free and redox active copper and iron have been detected in samples of advanced atherosclerotic plaque (Smith *et al.*, 1992; Lamb *et al.*, 1995; Swain & Gutteridge, 1995; Stadler *et al.*, 2004). Iron, in particular, has been found colocalized with ceroid (Lee *et al.*, 1998) and apoptotic foam cells (Yuan, 1999). However, many researchers question the existence of free metals during early stages of the disease. The finding that various metal carrying proteins remain capable of promoting LDL oxidation under appropriate conditions may help to alleviate such concerns.

Physiological levels of the blood's major copper transporter, ceruloplasmin, have been shown to induce oxidation *in vitro* (Ehrenwald *et al.*, 1994). Intact ceruloplasmin is required for this oxidation and it appears to involve only one of the seven bound copper ions (Ehrenwald *et al.*, 1994; Mukhopadhyay *et al.*, 1997). Ceruloplasmin also requires a reducing environment and, while LDL fulfils this prerequisite in phosphate buffered saline (PBS), cells or a superoxide generating system must provide it in culture medium (Fox *et al.*, 2000). Of direct relevance to atherosclerosis, ceruloplasmin is synthesized by activated macrophages (Ehrenwald & Fox, 1996; Mazumder *et al.*, 1997) and has been immunohistochemically identified in samples of atherosclerotic plaque (Fox *et al.*, 2000). In addition, ceruloplasmin becomes more catalytically active under acidic conditions (Lamb & Leake, 1994b) and the atherosclerotic plaque is considered to provide just such an environment (Leake, 1997).

Iron carrying proteins may also contribute to LDL oxidation, with ferritin and transferrin both exhibiting pro-oxidant properties under conditions that promote iron release. Superoxide radicals and an acidic pH are each suggested to provide this kind of environment (Abdalla *et al.*, 1992; Lamb & Leake, 1994a). Another iron carrier, hemin, exhibits pro-oxidant activity even at neutral pH and in the presence of human serum (Tribble *et al.*, 1996; Camejo *et al.*, 1998). Such activity has been attributed to hemin's ability to intercalate with the surface monolayer of LDL. Co-incubation of this haemoglobin degradation product with hydrogen peroxide further accelerates LDL oxidation, presumably by inducing the release of iron from the hemin (Camejo *et al.*, 1998) (Balla *et al.*, 1991). Additional support for a pro-atherogenic effect of hemin arises from the discovery that atherosclerotic lesions are reduced in mice overexpressing the heme oxygenase enzyme (Ishikawa *et al.*, 2001).

An alternative source of metal may be provided by macrophages that have previously phagocytosed iron-rich cellular debris and erythrocytes. Exocytosis of this lysosomal iron after macrophage entry into the atherosclerotic lesion would then ensure that the iron was readily available for promoting LDL oxidation (Yuan *et al.*, 1995).

Although these studies once again support the potential for metal-mediated LDL oxidation, they fail to confirm the significance of such reactions in humans. Fu *et al.* (1998) examined protein extracted from atherosclerotic plaque and did find evidence of hydroxyl radical attack and Fenton chemistry. However, only advanced lesions were sampled in this study. By contrast, Leeuwenburgh *et al.* (1997b) analyzed protein isolated from lesions of all stages. They observed patterns specific to metal-catalyzed

oxidation only in the advanced plaque, suggesting that metals are more important at later stages of the disease.

Copper levels are significantly elevated in people afflicted with Wilson's disease but this increase fails to correlate with atherosclerosis (Heinecke, 1999). Nevertheless, it is possible that an increased atherosclerotic risk is masked by the decrease in ceruloplasmin that is also associated with these individuals (Heinecke, 1999). Epidemiological studies correlating atherosclerotic risk to iron levels have also proved contradictory, even when studying a disease associated with excessive iron deposition like haemochromatosis (You & Wang, 2005). Reasons for this disparity are unclear but may be due, at least partly, to an inappropriate choice of markers. For instance, combining measurements of elevated LDL with transferrin saturation is suggested to provide a more accurate indication of atherosclerotic risk than either parameter alone (Wells *et al.*, 2004). Likewise, the labile iron pool may represent a more relevant source of the iron available for promoting LDL oxidation than many other iron markers. In support of this, Gackowski *et al*. (2001) noted a significant correlation between atherosclerotic risk and elevated levels of the labile iron pool in lymphocytes.

ii) Lipoxygenase-Mediated LDL Oxidation

Cells, particularly monocytes/macrophages, may promote LDL oxidation by pathways that are independent of their metal reducing activities. Lipoxygenases (LOs) selectively catalyze the peroxidation of PUFAs (Cathcart & Folcik, 2000) and, as such, this class of enzymes has long been studied for their role in atherogenesis. It is now generally accepted that 5LO does not significantly participate in macrophage-mediated LDL oxidation but 15LO remains a potential candidate (Chisolm *et al.*, 1999). The intracellular nature of 15LO has, in turn, raised some important questions about its relevance in LDL oxidation. Several mechanisms of action have been proposed to overcome this issue, including the transfer of cell-derived 15LO products to LDL (Cathcart & Folcik, 2000). A direct interaction between 15LO and LDL might also occur after lipoprotein uptake. Alternatively, by altering the balance of PUFA oxidation products, this enzyme may affect signal transduction and/or alternative metabolic pathways involved in LDL oxidation. Obtaining definitive evidence of LO-mediated LDL oxidation in cell culture is hindered by the fact that, similar to many superoxide inhibitors, LO inhibitors often possess additional non-specific antioxidant functions

(Chisolm *et al.*, 1999). Nevertheless, mouse models have shown that an absence of functional 12/15LO activity significantly reduces the extent of atherosclerosis compared to controls (Cyrus *et al.*, 1999; George *et al.*, 2001). 15LO has also been detected, colocalized with oxLDL, in rabbit and human atherosclerotic plaque (Yla-Herttuala *et al.*, 1990; Yla-Herttuala *et al.*, 1991). Of course, colocalization does not necessarily indicate a causative role for LO in LDL oxidation. It remains possible that the oxLDL is present first and merely induces an upregulation of macrophage 15LO levels (Garner & Jessup, 1996). More direct evidence for LO-mediated oxidation is therefore provided by identification of 15LO-associated stereospecific chirality in lipids extracted from human atherosclerotic lesions (Folcik *et al.*, 1995). While another study detected significant chirality only in early lesions (Kuhn *et al.*, 1997), this finding has since been attributed simply to a lower sample size (Cathcart & Folcik, 2000). Nevertheless, these results must still be interpreted with caution, because stereospecific lipid peroxidation products may be generated via other enzymatic pathways (Heinecke, 1998).

iii) Nitric Oxide- and Hypochlorite-Mediated LDL Oxidation

The subendothelial space of the vessel wall is reported to contain relatively high concentrations of nitric oxide (Westhuyzen, 1997). While nitric oxide, itself, is unable to oxidize LDL under physiological conditions, several of the reactive nitrogen species (RNS) derived from this radical are known to be potent oxidants (Carr *et al.*, 2000a). Peroxynitrite, in particular, is formed by the reaction between superoxide and nitric oxide (Squadrito & Pryor, 1998) and has been shown both to nitrate the tyrosyl residue on the apoB100 of LDL and to promote lipid peroxidation *in vitro* (Hazen *et al.*, 1999). By contrast, cell culture studies have demonstrated an overall anti-atherogenic effect of nitric oxide in mouse macrophages (Jessup *et al.*, 1992; Jessup & Dean, 1993; Niu *et al.*, 2000). Inducible nitric oxide synthase (iNOS) (Luoma *et al.*, 1998) and 3-nitrotyrosine (Leeuwenburgh *et al.*, 1997a; Ischiropoulos, 1998) have been detected in human atherosclerotic plaque. However, a direct comparison between tyrosine nitration and oxidation has revealed that the former accounts for less than 3% of tyrosine modifications in atherosclerotic lesions (Morton *et al.*, 2003). Oxidative products like dityrosine were found to be considerably more abundant.

Furthermore, 3-nitrotyrosine is not a specific marker of peroxynitrite attack. The myeloperoxidase (MPO) enzyme can also nitrate tyrosyl residues, as confirmed by both

in vitro and mouse *in vivo* studies (Hazen *et al.*, 1999; Carr *et al.*, 2000a; Gaut *et al.*, 2002). MPO is a heme protein of phagocytic cells that is detectable during inflammation (Heinecke, 2002) and, in addition to promoting nitration, this enzyme also catalyzes other reactions. Incubating MPO with hydrogen peroxide and tyrosine results in the production of tyrosyl radicals. These radicals can then induce lipid peroxidation (Savenkova *et al.*, 1994) or dityrosine formation and subsequent cross-linkage of proteins (Heinecke *et al.*, 1993b). Alternatively, incubating MPO in the presence of hydrogen peroxide and chlorine ions generates hypochlorite, a reactive product known to oxidize cholesterol to chlorohydrins and to particularly attack protein (Berliner & Heinecke, 1996). Not surprisingly, therefore, apoB100 is the primary target of hypochlorite-mediated LDL oxidation (Hazell & Stocker, 1993). The ensuing lipid peroxidation is attributed to secondary reactions between chloramine break-down products and the lipid (Hazell *et al.*, 1999). MPO's atherogenic effect is likely to be aided by its ability to bind LDL, thereby permitting site-directed oxidation of the lipoprotein and reducing the effectiveness of antioxidants (Carr *et al.*, 2000b). The potential for this interaction *in vivo* is high because active MPO has been isolated from atherosclerotic plaque, where it is found predominantly colocalized with macrophages in the shoulder region of the fibrous cap or adjacent to cholesterol clefts in lipid-rich areas and microvessels (Daugherty *et al.*, 1994; Sugiyama *et al.*, 2001). Moreover, markers of MPO-mediated oxidation have been detected on lipoproteins isolated from human atherosclerotic lesions. These markers include 3-chlorotyrosine (Hazen & Heinecke, 1997), protein-bound p-hydroxyphenylacetaldehyde (Hazen *et al.*, 2000) and even plaque protein that cross-reacts with antibodies raised against hypochlorite-modified LDL (Hazell *et al.*, 1996). In contrast to markers of metal-catalyzed oxidation, MPO markers appear to be present at all stages of lesion development, suggesting a role for MPO from the initial fatty streak to the advanced plaque (Leeuwenburgh *et al.*, 1997b; Heinecke, 1998). Paradoxically, mice deficient in MPO develop larger atherosclerotic lesions than controls (Brennan & Hazen, 2003). The relevance of this finding is doubtful because lesions from control mice contain no detectable MPO or 3-chlorotyrosine anyway (Brennan & Hazen, 2003). Corresponding human studies may be more appropriate and have, in fact, highlighted a correlation between patients with reduced MPO expression and a decreased risk of cardiovascular disease (Nikpoor *et al.*, 2001).

An alternative pathway for LDL oxidation involves endocytosis of minimally modified LDL (mmLDL) by the macrophage LDL receptor, with subsequent oxidation only upon entering the acidic environment of endosomes and lysosomes (Jessup & Kritharides, 2000).

Thus, multiple pathways are likely to be involved in LDL oxidation *in vivo*, with some displaying a cell- and/or stage-specificity. Whichever mechanism(s) are involved in LDL modification *in vivo*, the end product is ultimately a heterogeneous population of LDL that is oxidized to varying degrees. At one end of the spectrum is minimally modified LDL (mmLDL) and, as the name suggests, such particles have only been oxidized to a small extent. At the other end of the spectrum, by contrast, is the heavily oxLDL that has been extensively modified.

1.2.3 Atherogenicity of oxLDL

As a heterogeneous population, oxLDL is able to promote a range of atherogenic effects. All contribute further to the development of atherosclerosis but the exact biological activity differs depending on the extent of oxidation. Thus, the properties of mmLDL differ from those of the more heavily oxLDL, with the former still being recognized by the LDL receptor but also playing a critical role in the induction of various pro-inflammatory cytokines. Continued oxidation reduces this activity and ultimately produces a particle that is both rapidly taken up by scavenger receptors and is cytotoxic to cells (Berliner *et al.*, 1995).

OxLDL-mediated cytotoxicity is directed not only towards oxidizing macrophage cells themselves (Marchant *et al.*, 1996) but also towards other cells subsequently exposed to the oxLDL. Cell types tested under these conditions include SMCs (Nishio *et al.*, 1996), endothelial cells (Li *et al.*, 1998a), macrophages (Kinscherf *et al.*, 1998), T cells (Alcouffe *et al.*, 1999) and fibroblasts (Chisolm *et al.*, 1994). Depending on the cell type and extent of damage, this cell death may occur via caspase-dependent or -independent apoptosis or via necrosis (Vicca *et al.*, 2000; Yuan *et al.*, 2000; Asmis & Begley, 2003; Baird *et al.*, 2004). While the cytotoxic activity of oxLDL is predominantly attributed to 7β-hydroperoxy-cholesterol (Chisolm *et al.*, 1994; Colles *et al.*, 1996), other oxysterols like 7-ketocholesterol and 7β-hydroxycholesterol can also contribute to cell death (Colles *et al.*, 1996; Nishio *et al.*, 1996). These cholesterol oxidation products are reported to destabilize macrophage lysosomes, leading to

leakage of lysosomal contents and subsequent induction of apoptosis or necrosis (Yuan *et al.*, 2000). 7β-hydroperoxy-cholesterol decomposition further contributes to oxysterol toxicity due to the formation of reactive alkoxyl, peroxyl and lipid radicals that can propagate peroxidation of cellular lipids (Coffey *et al.*, 1995). Other hydroperoxides and their aldehyde break-down products have also been suggested to contribute to the cytotoxicity of oxLDL (Siow *et al.*, 1999; Uchida, 2000; Choudhary *et al.*, 2002), as have phospholipid hydrolysis products like lysophosphatidylcholine (Carpenter *et al.*, 2001).

Components of oxLDL and ceroid are known to accumulate in macrophage lysosomes (Mander *et al.*, 1994; Brown *et al.*, 2000; Jessup & Kritharides, 2000) and this is caused, at least in part, by inactivation of some of the lysosomal proteases and hydrolases (Kritharides *et al.*, 1998; Li *et al.*, 1998b). This accumulation clearly contributes to cytotoxicity but it also has the potential to impair other cellular activities, including endocytic and secretory macrophage functions (Bolton *et al.*, 1997).

Several pro-atherogenic consequences arise from oxLDL-mediated cytotoxicity. In particular, damaged endothelial cells may enhance entry of LDL and/or monocytes into the arterial intima (Steinberg *et al.*, 1989) while macrophage death may contribute to growth of the necrotic core (Bjorkerud & Bjorkerud, 1996). Plaque instability may be promoted at later stages of atherosclerosis due to endothelial denudation (Steinberg *et al.*, 1989) or macrophage and SMC death (section 1.2.1.3).

OxLDL promotes atherogenesis via additional non-cytotoxic pathways, particularly when present as mmLDL. It is known to act as a chemoattractant towards both T cells and monocytes (Young & McEneny, 2001) and, while some of this activity is directly attributable to mmLDL, it is further aided by the mmLDL-mediated induction of chemoattractant cytokines like P-selectin, GRO, VCAM-1 and MCP-1 (Berliner *et al.*, 1995; Berliner & Heinecke, 1996). Once recruited to the intima, monocytes are then subject to an additional array of actions mediated by oxidized components of LDL. In particular, monocyte differentiation is promoted by the oxLDL-induced release of endothelial-derived M-CSF (Rajavashisth *et al.*, 1990). Subsequent macrophage proliferation (Martens *et al.*, 1999) and retention in the intima (Quinn *et al.*, 1987) are also enhanced by this modified lipoprotein. Likewise, macrophage cholesterol metabolism is influenced by oxLDL at all stages, from uptake to degradation (as described above) and even efflux. While evidence for uptake is provided by increased

SRA and CD36 expression (Nakagawa *et al.*, 1998; Glass & Witztum, 2001), cholesterol efflux studies have proved more contradictory. One group observed increased cholesterol export due to the oxLDL-mediated upregulation of ATP binding cassette transporter-1 (ABC1) (Tang *et al.*, 2004) but others have noted inhibition of efflux (Kritharides *et al.*, 1995b; Gelissen *et al.*, 1996). Such disparity may be due to cell-specific effects or to differences in the nature of the oxLDL being studied.

The pro-atherogenic activity of oxLDL also extends to other cells, ranging from enhanced SMC proliferation (Auge *et al.*, 2002) and SMC migration (Gorog & Kovacs, 1998) to activation of T lymphocytes (Frostegard *et al.*, 1992) but inhibition of endothelial cell migration (Murugesan & Fox, 1996). Conversely, oxLDL may promote endothelial proliferation via upregulation of vascular endothelial growth factor (VEGF) expression (Inoue *et al.*, 2001). This proliferation exerts an anti-atherogenic effect early in atherosclerosis but is pro-atherogenic at later stages due to microvessel formation. OxLDL further influences endothelial cell activity by inhibiting nitric oxide production (Huang *et al.*, 1999) but stimulating endothelin synthesis (Berliner & Heinecke, 1996). The net result is a loss of vasodilatory activity in the artery wall.

Likewise, oxLDL adversely affects plaque stability. It promotes a net increase in macrophage-mediated matrix degradation by down-regulating the tissue inhibitor of metalloproteinase-1 (TIMP-1) but upregulating matrix metalloproteinase-9 (MMP-9) (Xu *et al.*, 1998). OxLDL also stimulates the production of tissue factor (Berliner *et al.*, 1995) and promotes platelet aggregation (Volf *et al.*, 2000), further raising the likelihood of thrombus formation.

It is therefore clear that oxLDL induces a wide range of biological activities. While most of these activities are attributed to its oxidized phospholipids, including lysophosphatidylcholine (Chisolm & Chai, 2000; Subbanagounder *et al.*, 2000), other components of oxLDL are also postulated to play a contributory role. For instance, oxLDL's protein moiety appears to stimulate interleukin-1 (IL-1) production (Lipton *et al.*, 1995) and respiratory burst activity in macrophages (Nguyen-Khoa *et al.*, 1999) while lipid hydroperoxides inhibit nitric oxide synthesis (Huang *et al.*, 1999). Furthermore, inhibition of cholesterol efflux is primarily ascribed to 7-ketocholesterol and the accumulation of undegradable cholesteryl esters in lysosomes (Jessup *et al.*, 2004).

OxLDL's capacity to influence so many seemingly disparate biological functions is ultimately attributed to an increase in secondary messengers, like cyclic adenosine

monophosphate (cAMP) (Parhami *et al.*, 1993), and to the induction of various cytokines and signal transduction pathways (Berliner & Heinecke, 1996). Signal transduction occurs through the oxLDL-mediated activation of protein kinase C (PKC), nuclear factor-κβ (NF-κβ), activator protein-1 (AP-1) and peroxisome proliferator-activated receptor-γ (PPARγ) (Parhami *et al.*, 1993; Berliner & Heinecke, 1996; Li *et al.*, 1998a; Nagy *et al.*, 1998; Tontonoz *et al.*, 1998; Inoue *et al.*, 2001; Whatling *et al.*, 2004).

1.2.4 Inflammation in Atherosclerosis

The atherosclerotic plaque is clearly an environment of chronic inflammation. T lymphocytes and macrophages are numerous and activated, as demonstrated by the immunohistochemical detection of pro-inflammatory cytokines in plaque lesions and by the expression of various receptors and major histocompatability complexes (MHCs) on cell surfaces (Libby & Hansson, 1991; Wick *et al.*, 1995). These T cells are primarily of the pro-inflammatory Th1 type rather than the anti-inflammatory Th2 cells (Frostegard *et al.*, 1999) and it is suggested that oxLDL may help drive the T lymphocytes in this direction (Varadhachary *et al.*, 2001). The diminished anti-inflammatory response observed in monocytes/macrophages isolated from acute coronary syndrome patients ensures further enhancement of the pro-atherogenic environment (van Haelst *et al.*, 2004).

Antibodies represent another aspect of the inflammatory reaction and, given oxLDL's highly immunogenic nature (Horkko *et al.*, 2000), it is not surprising that autoantibodies to oxLDL have been detected in the plasma of atherosclerotic patients (Salonen *et al.*, 1992; Shaw *et al.*, 2001). However, the relationship between antibody levels and atherosclerotic risk remains complex and often contradictory. Despite this complexity, Hulthe (2004) suggests that the extent of atherosclerosis generally correlates positively with IgG titres but negatively with IgM titres.

Studies of the complement system have proved similarly complex. Rabbits deficient in complement C6 were found to develop significantly less atherosclerosis than wild-type controls (Schmidt *et al.*, 1998) but a deficiency of complement C5 in mice afforded no protection against the disease (Patel *et al.*, 2001). Rabbits may be more representative of humans in this case because the C5b-9 terminal complement complex has been detected in human atherosclerotic plaque, colocalized with apoptotic cells

(Niculescu *et al.*, 2004). Furthermore, elevated levels of C5a are associated with an increased risk of future adverse cardiovascular events in patients with advanced atherosclerosis (Speidl *et al.*, 2005).

Various infectious agents have also been implicated in atherosclerosis, most notably *Chlamydia pneumonia*, *Helicobacter pylori* and cytomegalovirus (Torgano *et al.*, 1999) (Osterud & Bjorklid, 2003). Whether these infectious agent(s) promote atherosclerosis through some direct mechanism or indirectly via the promotion of an inflammatory state is currently unclear. Nevertheless, it should be noted that lipopolysaccharide (LPS) is capable of enhancing both cell- and copper-mediated LDL oxidation (Maziere *et al.*, 1999).

The detection of numerous inflammatory markers in patients provides additional support for the inflammatory nature of the atherosclerotic lesion (Erren *et al.*, 1999; Van Lente, 2000). Some markers, particularly heat shock protein 65 (hsp65) (Xu *et al.*, 1993) and C reactive protein (CRP) (Patrick & Uzick, 2001; Wilson *et al.*, 2006), even correlate with the extent of atherosclerosis. Rather than serving as simple diagnostic markers, these two parameters are also suggested to be directly pro-atherogenic (Wick *et al.*, 1995; Wilson *et al.*, 2006).

1.2.4.1 Interferon-γ

IFN-γ is currently reported to regulate over 500 genes (Leon & Zuckerman, 2005), ensuring that the effects of this cytokine are complex, multifactorial and associated with both pro- and anti-atherogenic activities. Potential pro-atherogenic functions include the IFN-γ-mediated upregulation of antigen presenting components like MHCs (Hansson *et al.*, 2006); activation of macrophages and stimulation of the respiratory burst (Hansson *et al.*, 2006); and induction of ceruloplasmin synthesis (Mazumder *et al.*, 1997). Its ability to promote T lymphocyte and monocyte recruitment is also considered pro-atherogenic. The former involves an enhanced release of endothelial-derived T cell α-chemoattractant (Cole *et al.*, 1998) while the latter is associated with increased expression of the VCAM-1, ICAM-1 and MCP-1 chemokines (Leon & Zuckerman, 2005). Conversely, IFN-γ has been reported to inhibit induction of E-selectin, P-selectin, PECAM-1 and the MCP-1 receptor, CCR2 (Leon & Zuckerman, 2005). IFN-γ's inhibition of 15-LO synthesis is, likewise, described as antiatherogenic (Folcik *et al.*, 1997).

The pro-atherogenic properties of IFN-γ also extend to plaque instability and thrombus formation. Expression of the multifunctional CD40 and its ligand (CD40L) are enhanced in the presence of IFN-γ (Schonbeck & Libby, 2001), as are cathepsin S (Beers *et al.*, 2003), MMP-1 (Anderson *et al.*, 2002; Zhou *et al.*, 2003) and the pro-coagulant tissue factor (Collins *et al.*, 1995). By contrast, IFN-γ is reported to inhibit synthesis of MMP-9 (Zhou *et al.*, 2003) and cathepsin L (Beers *et al.*, 2003). Nevertheless, because IFN-γ also inhibits SMC proliferation (Hansson *et al.*, 1989; Warner *et al.*, 1989), collagen synthesis (Weitkamp *et al.*, 1999; Xu *et al.*, 2004) and α-actin expression (Hansson *et al.*, 1989), the net effect of this cytokine is likely to be one of plaque destabilization in advanced lesions.

The influence of IFN-γ on cholesterol metabolism is rather complex and, as such, the net result will depend on the balance between various conflicting actions. Although the human macrophage CD36 (Nakagawa *et al.*, 1998; Panousis & Zuckerman, 2000a) is down-regulated by IFN-γ, its SR-PSOX receptor is paradoxically upregulated (Wuttge *et al.*, 2004). Likewise, SRA expression is inhibited in macrophages (Geng & Hansson, 1992; Grewal *et al.*, 2001) but stimulated in monocytes (Grewal *et al.*, 2001) and SMCs (Li *et al.*, 1995). IFN-γ has been theorized to limit foam cell formation via its reduction of macrophage very low density lipoprotein (VLDL) receptor expression (Kosaka *et al.*, 2001), lipoprotein (a) (Lp(a)) expression (Skiba *et al.*, 1994), LDL receptor-related protein (LRP) activity (LaMarre *et al.*, 1993; Garner *et al.*, 1997a) and lipoprotein lipase (LPL) secretion (Jonasson *et al.*, 1990; Garner *et al.*, 1997a). Conversely, IFN-γ stimulates acyl-CoA: cholesterol-O-acyl-transferase (ACAT) activity (Panousis & Zuckerman, 2000a) and inhibits ABC1 expression (Panousis & Zuckerman, 2000b), apolipoprotein E (ApoE) production (Brand *et al.*, 1993; Garner *et al.*, 1997a) and cholesterol 27-hydroxylase production (Reiss *et al.*, 2004). These activities would all be expected to diminish cholesterol efflux, thereby promoting foam cell formation.

The stage of atherosclerosis may play an equally critical role in determining IFN-γ's overall effect. A prime example of this is highlighted by the inhibition of collagen synthesis and SMC proliferation, which would be considered anti-atherogenic during early atherosclerosis but pro-atherogenic in advanced plaques.

Attempts to address IFN-γ's pro- versus anti-atherogenic effect in cell culture or animal models have produced contradictory results. Mice engineered to lack either a functional IFN-γ receptor (Gupta *et al.*, 1997b) or gene (Buono *et al.*, 2003) developed

significantly less diet-induced atherosclerosis than controls, while the intraperitoneal administration of exogenous IFN-γ enhanced atherogenesis (Whitman *et al.*, 2000). This pro-atherogenic effect was observed in mice even in the absence of leukocytes (Tellides *et al.*, 2000). By contrast, rabbits subjected to intramuscular injections of IFN-γ exhibited a reduction in lesion size and frequency (Wilson *et al.*, 1990). On a cellular level, IFN-γ's net effect also appears anti-atherogenic (Fong *et al.*, 1994). It is suggested to inhibit mouse macrophage-mediated LDL oxidation by stimulating iNOS to generate nitric oxide (Jessup & Dean, 1993; Niu *et al.*, 2000). The protection against oxidative attack observed when incubating LDL with human macrophages, IFN-γ and tryptophan has been attributed to IFN-γ's ability to upregulate indoleamine 2,3-dioxygenase (IDO) (Christen *et al.*, 1994). This enzyme participates in tryptophan degradation and one of the final break-down products in the pathway, 3-hydroxyanthranilic acid (3-HAA), exhibits strong antioxidant activity (Christen *et al.*, 1990; Thomas *et al.*, 1996b). Likewise, the IFN-γ-mediated induction of heme oxygenase ultimately generates bilirubin, another compound with known antioxidant activity (Neuzil & Stocker, 1994; Bowry *et al.*, 1995).

1.3 Neopterin and 7,8-Dihydroneopterin

In addition to all the activities discussed above, IFN-γ is also responsible for stimulating the synthesis of 7,8-dihydroneopterin (7,8-NP) and neopterin (Huber *et al.*, 1984). These molecules belong to a class of compounds called pteridines. All pteridines are characterized by a pyrazino-2,3-pyrimidine bicyclic nitrogen-containing ring system and can be further described as either conjugated or unconjugated, depending on the size of the substituent groups (Wachter *et al.*, 1992). Additional subclassification of unconjugated pterdines is based on their oxidation states, ranging from fully reduced tetrahydropterins to partially reduced dihydropterins and aromatic pterins (Oettl & Reibnegger, 2002). 7,8-NP is an unconjugated partially reduced dihydropterin while neopterin is one of its oxidation products.

1.3.1 Synthesis

All pteridines, including 7,8-NP and neopterin, are synthesized from a guanosine triphosphate (GTP) precursor (Figure 1.4). IFN-γ promotes this initial step, ensuring the

efficient conversion of GTP to 7,8-NP triphosphate via upregulation of GTP cyclohydrolase I expression (Werner *et al.*, 1990). Phosphatases then generate 7,8-NP by hydrolysing the 7,8-NP triphosphate (Wachter *et al.*, 1992). Neopterin may be produced either by oxidation of 7,8-NP triphosphate, with subsequent phosphatase-mediated hydrolysis of the newly formed neopterin triphosphate, or by oxidation of 7,8-NP (Hoffmann *et al.*, 2003).

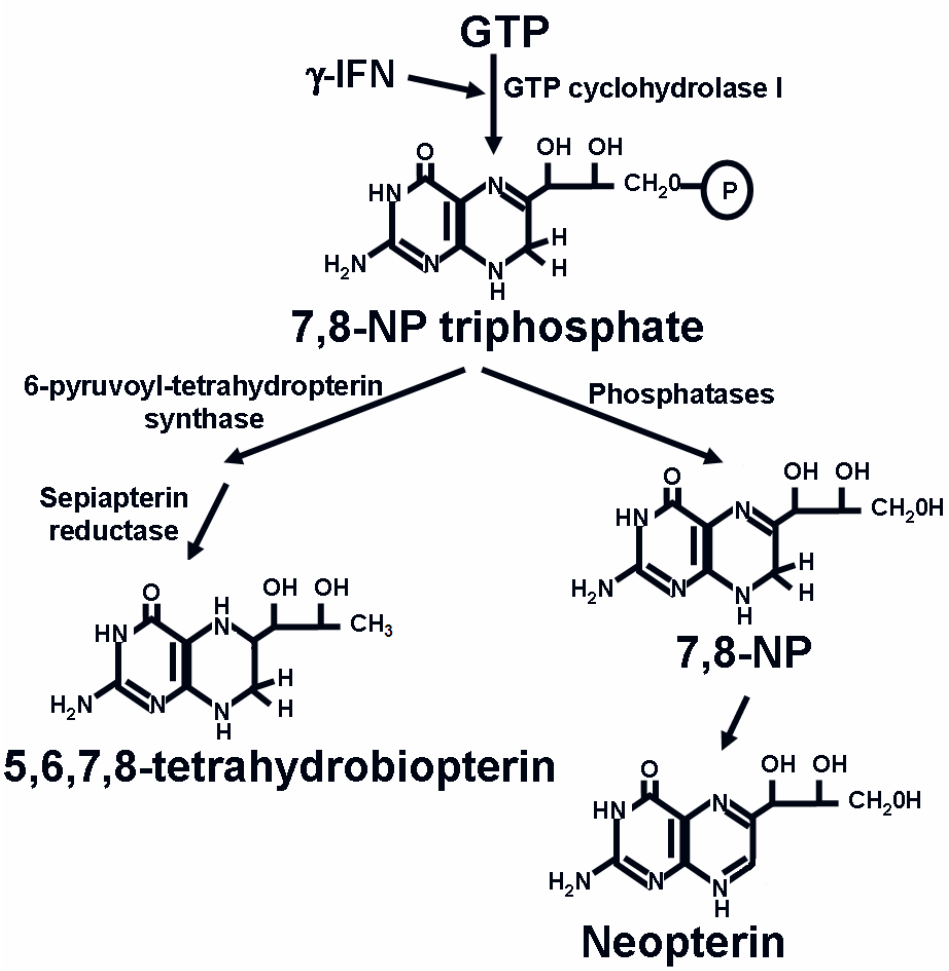


Figure 1.4 Biosynthetic pathway for 7,8-NP formation.
Adapted from Wachter *et al.* (1992).

An alternative pathway for 7,8-NP triphosphate generates a fully reduced pteridine, 5,6,7,8-tetrahydrobiopterin (BH_4), and requires the sequential action of 6-pyruvol tetrahydropterin synthase (6-PTPS) and sepiapterin reductase (Wachter *et al.*, 1992). Both enzymes are constitutively expressed but, in human macrophages, 6-PTPS activity is

very low (Werner *et al.*, 1990). This is attributed to a cell-specific transcriptional skip of the 23bp exon 3, thereby creating a premature stop codon in human macrophage 6-PTPS mRNA (Leitner *et al.*, 2003). The rate limiting effect of 6-PTPS ensures that 7,8-NP is synthesized in macrophages while BH_4 is the predominant product in cells like fibroblasts and T lymphocytes (Werner *et al.*, 1990). BH_4 serves as a cofactor not only for aromatic amino acid monoxygenases but also for nitric oxide synthase (Walter *et al.*, 2001). Thus, in contrast to other cells, nitric oxide production is not a significant consequence of the upregulation of GTP cyclohydrolase I in macrophages (Padgett & Pruett, 1992; Thomas *et al.*, 1994c).

Although IFN-γ is the strongest inducer of 7,8-NP/neopterin production in macrophages, co-stimulation with tumour necrosis factor-α (TNFα), dexamethasone or LPS has also been shown to enhance synthesis (Werner-Felmayer *et al.*, 1990; Werner-Felmayer *et al.*, 1995). Likewise, neopterin levels are elevated upon co-incubation of IFN-γ with increasing concentrations of extracellular tryptophan (Werner *et al.*, 1989; Werner-Felmayer *et al.*, 1990). By contrast, neopterin production was found to be down-regulated after the exposure of THP-1 monocytes to α-melanocyte-stimulating hormone (α-MSH) (Rajora *et al.*, 1996), transferrin-bound or free iron (Weiss *et al.*, 1992) or histamine (Gruber *et al.*, 2000). While the latter is suggested to involve a histamine-H_2-receptor interaction (Gruber *et al.*, 2000), the effect of iron has more specifically been ascribed to transcriptional and post-transcriptional disruption of GTP cyclohydrolase I expression (Oexle *et al.*, 2003). More indirect influences on neopterin synthesis are observed in cultures of peripheral blood mononuclear cells (PBMCs), with the pro-inflammatory IL-12 cytokine enhancing T lymphocyte activity and IFN-γ release (Weiss *et al.*, 1999). The anti-inflammatory IL-4 and IL-10 cytokines inhibit these processes, and there is some suggestion that they may also directly down-regulate IFN-γ's effect on macrophages (Weiss *et al.*, 1999).

These findings are all of potential relevance to atherosclerosis because histamine (Sasaguri & Tanimoto, 2004), iron (Smith *et al.*, 1992; Swain & Gutteridge, 1995; Lee *et al.*, 1998; Yuan, 1999) and cytokines (Geng *et al.*, 1995; Berliner & Heinecke, 1996; Frostegard *et al.*, 1999) have all been detected in lesions. Furthermore, serum neopterin levels appear to be elevated in patients with atherosclerosis (Tatzber *et al.*, 1991; Weiss *et al.*, 1994; Erren *et al.*, 1999).

1.3.2 Neopterin as a Marker of Inflammation

Neopterin levels in serum, urine and/or cerebrospinal fluid are also elevated in diseases ranging from endometriosis (Kondera-Anasz *et al.*, 2004) and various cancers (Murr *et al.*, 1999) to neurodegenerative and autoimmune disorders (Wirleitner *et al.*, 2005). In many cases, the disease stage and/or disease prognosis have been correlated to the extent of neopterin production (Hamerlinck, 1999) (Hoffmann *et al.*, 2003).

Elevated neopterin levels can often be associated with the severity of viral, bacterial, protozoic, fungal and parasitic infections too (Hamerlinck, 1999; Hoffmann *et al.*, 2003). While much of the viral research has focussed on the predictive value of neopterin in human immunodeficiency virus (HIV) patients (Tsoukas & Bernard, 1994), neopterin has also been seriously considered as a marker for other viral infections. Severe acute respiratory syndrome (SARS) (Zheng *et al.*, 2005) and hepatitis B (Kalkan *et al.*, 2005) are just two examples.

Neopterin and immune activation are, without a doubt, closely correlated. Thus, it is not surprising that elevated neopterin also serves as a useful marker of infection and/or allograft rejection following organ transplantation (Hoffmann *et al.*, 2003). Of particular significance, neopterin measurements were indicative of transplant rejection an average of two days prior to confirmation by conventional diagnosis (Hamerlinck, 1999).

1.3.3 Physiological Function of Neopterin and 7,8-NP

Although neopterin's suitability as a marker of inflammation has long been acknowledged, the biological function of this pteridine and its reduced counterpart remains an issue of contention. Numerous *in vitro* studies have provided evidence of both pro- and anti-oxidative activities as well as roles in apoptosis, signal transduction and gene expression.

1.3.3.1 Enzyme Inhibition

Neopterin was initially hypothesized to act as an antimicrobial agent via inhibition of folate biosynthesis (Nathan, 1986). However, in the absence of evidence for a direct effect of neopterin on bacterial cultures, support for this theory has since waned (Hoffmann *et al.*, 2003). By contrast, neopterin has been specifically confirmed to inhibit the activity of at least two other enzymes *in vitro* – the superoxide generators,

NADPH oxidase and xanthine oxidase. Inhibition of the former occurs via competitive inhibition of the NADPH substrate (Kojima *et al.*, 1993) while inhibition of the latter involves a reversible, non-competitive mechanism (Oettl *et al.*, 1997; Oettl & Reibnegger, 1999). It has also recently been suggested that neopterin may inhibit myeloperoxidase, leading to an increase in singlet oxygen but a decrease in all other reactive oxygen species (ROS) (Razumovitch *et al.*, 2004).

The *in vivo* relevance of such findings has been questioned because of the micromolar quantities of neopterin required for enzyme inhibition. Oettl & Reibnegger (2002) counteract this argument by noting that high concentrations of neopterin may accumulate intracellularly and in close proximity to enzymes like NADPH oxidase. Furthermore, the intravenous administration of neopterin was shown to inhibit ischemic paw edema in mice and gastric ischemic injury in rats (Icho *et al.*, 1995). As superoxide is suggested to significantly contribute to the development of both diseases, neopterin may provide protection by directly inhibiting the superoxide generating enzymes. Alternatively, it may enter cells and be intracellularly reduced to dihydroneopterin or tetrahydroneopterin, both of which have strong radical scavenging activity (Kojima *et al.*, 1992; Weiss *et al.*, 1993; Icho *et al.*, 1995; Oettl & Reibnegger, 2002).

1.3.3.2 Radical Scavenging

7,8-NP is unable to directly inhibit NADPH oxidase activity but still limits radical production by scavenging the superoxide generated by this enzyme (Oettl *et al.*, 1997). This radical scavenging activity was initially demonstrated using hydroxyl radicals generated from an iron/hydrogen peroxide Fenton system (Heales *et al.*, 1988). Additional confirmation arose from 7,8-NP's ability to inhibit the luminol-dependent chemiluminescence induced by hydrogen peroxide, oxidase systems, the chloramine T free radical generator and the SIN-1 nitric oxide generator (Weiss *et al.*, 1993; Shen, 1994; Reibnegger *et al.*, 1995; Murr *et al.*, 1996). Some of the reduced pterins tested in the Shen (1994) study were even found to be more effective than the classical antioxidants, ascorbic acid and glutathione. Likewise, spin trap studies yielded a rate constant of 10^7 $M^{-1}s^{-1}$ for the reaction between 7,8-NP and peroxyl radicals (Oettl *et al.*, 1997). This is close to the rate constant for the reaction between peroxyl radicals and α-tocopherol. 7,8-NP is also an efficient scavenger of non-physiological radicals

like 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-di-[3-ethylbenzthaizoline sulphonate] ($ABTS^{+}$) (Oettl & Reibnegger, 2002).

On a larger scale, the radical scavenging activity of 7,8-NP has been shown to protect *Escherichia coli* from chloramine T- and hypochlorite-induced growth inhibition and cytotoxicity (Weiss *et al.*, 1993; Horejsi *et al.*, 1996; Wede *et al.*, 1999). Similarly, radical scavenging explains the inhibition of lipid peroxidation observed for both linoleate and native LDL in the presence of 7,8-NP (Gieseg *et al.*, 1995; Gieseg & Cato, 2003; Gieseg *et al.*, 2003; Greilberger *et al.*, 2004). This inhibition occurred whether the oxidative stress was derived from copper ions or the peroxyl radical generator, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). Low micromolar concentrations of 7,8-NP were further found to inhibit ROS-mediated damage to a variety of other substrates, including bovine serum albumin (BSA) (Duggan *et al.*, 2001), erythrocytes (Gieseg *et al.*, 2001a) and the U937 monocytic cell line (Gieseg *et al.*, 2001b; Duggan *et al.*, 2002). These studies noted protection against damage that ranged from dityrosine and protein hydroperoxide formation to haemolysis, cell death and thiol oxidation. The 7,8-NP-mediated inhibition of protein hydroperoxide formation will be discussed in greater detail in chapter 5. It is worth noting now, however, that although 7,8-NP has been shown to decompose protein hydroperoxides on BSA, the degradation half-life is 120 minutes (Duggan *et al.*, 2001). Thus, 7,8-NP's scavenging activity is a more significant form of protection against the AAPH-mediated peroxidation of BSA. Similarly, 7,8-NP appears to prevent copper- and AAPH-induced protein hydroperoxide formation on LDL by scavenging the lipid-derived radicals that are suggested to promote protein peroxidation (Gieseg *et al.*, 2003).

7,8-NP also appears to efficiently scavenge reactive nitrogen species (RNS), inhibiting 3-nitrotyrosine formation in a simple system of 7,8-NP, tyrosine and peroxynitrite (Widner *et al.*, 1998; Oettl *et al.*, 2004).

1.3.3.3 Pro-oxidant/Antioxidant Balance and Apoptosis

Although 7,8-NP is clearly a potent radical scavenger, under certain circumstances this pteridine can also exhibit pro-oxidant activity. LDL oxidation serves as a useful model of this pro-oxidant/antioxidant balance because co-incubating 7,8-NP with copper and native LDL is strongly antioxidative. However, a 20 minute pre-incubation was found to inhibit LDL conjugated diene formation only after an initial 30 minutes of weak but

significant pro-oxidant activity (Herpfer *et al.*, 2002). This result was attributed to the 7,8-NP-mediated reduction of copper to its redox active form, with antioxidant activity then dominating reactions once the reduction was complete. It is therefore unsurprising that 7,8-NP has been directly confirmed to reduce another transition metal, iron, to the redox active ferrous ion state (Wirleitner *et al.*, 2005). 7,8-NP's strong reducing activity is also suggested to promote the oxidation of both minimally and moderately oxidized LDL in the presence or absence of copper ions (Greilberger *et al.*, 2004).

The fully reduced tetrahydrobiopterins have been shown to generate superoxide upon autoxidation (Walter *et al.*, 2001). Autoxidation of the partially reduced 7,8-NP is expected to yield similarly reactive products. This provides an alternative pathway for 7,8-NP-mediated oxidative stress and is supported by the work of Oettl *et al.* (1999) and Wirleitner *et al.* (2001). These groups used two different detection systems to confirm the formation of oxygen radicals in solutions containing 7,8-NP, and noted that radical production was further enhanced in the presence of transition metals. Additional support for radical production arises from the detection of pterin-derived radicals upon the co-incubation of 7,8-NP with SIN-1 and the TMIO spin trap (Oettl *et al.*, 2004).

These reactions highlight a parallel to the pro-oxidant/antioxidant balance of ascorbic acid and potentially explain the biphasic effects observed in some 7,8-NP studies. This pteridine inhibits hydrogen peroxide-induced chemiluminescence at lower concentrations but, by 5mM 7,8-NP, an enhancing effect is observed (Murr *et al.*, 1996). Likewise, micromolar concentrations of 7,8-NP generally inhibit cell death. Anti-Fas-induced apoptosis was inhibited in Jurkat T cells under these conditions (Wirleitner *et al.*, 1998), as was TNFα-induced apoptosis (Baier-Bitterlich *et al.*, 1995) and oxLDL- and AAPH-induced death (Baird *et al.*, 2005) in U937 cells. In the absence of any other apoptotic inducers, micromolar quantities of 7,8-NP neither promote nor inhibit death in a range of neuronal cells (Spottl *et al.*, 2000; Enzinger *et al.*, 2002b), monocytes (Baird *et al.*, 2005) and T lymphocytes (Wirleitner *et al.*, 1998). By contrast, 5mM 7,8-NP alone has been shown to directly promote apoptosis in astrocyte-like cells (Spottl *et al.*, 2000), neuronal cells (Spottl *et al.*, 2000; Enzinger *et al.*, 2001; Enzinger *et al.*, 2002b) and T lymphocytes (Baier-Bitterlich *et al.*, 1996a; Wirleitner *et al.*, 1998; Wirleitner *et al.*, 2001; Enzinger *et al.*, 2002a). Apoptosis in the U937 (Baier-Bitterlich *et al.*, 1995) and rat PC12 (Enzinger *et al.*, 2001) cells appears to be further enhanced by co-incubating 5mM 7,8-NP with TNFα.

A few exceptions to this biphasic trend do exist, with micromolar 7,8-NP concentrations inducing apoptosis in both the rat alveolar epithelial L2 cell line (Schobersberger *et al.*, 1996) and in human peripheral blood T cells (Wirleitner *et al.*, 2003). Nevertheless, this susceptibility has been attributed to species-, cell- and tumour-specific differences.

Antioxidants ranging from N-acetylcysteine (NAC) to pyrollidine dithiocarbamate (PDTC), catalase and SOD have been demonstrated to significantly inhibit 7,8-NP-mediated apoptosis (Baier-Bitterlich *et al.*, 1995; Baier-Bitterlich *et al.*, 1996a; Wirleitner *et al.*, 1998; Spottl *et al.*, 2000; Enzinger *et al.*, 2002b; Wirleitner *et al.*, 2003). It therefore appears that the presence of excess 7,8-NP results in ROS generation, disrupting the oxidant/antioxidant balance and ultimately leading to cell death.

In contrast to 7,8-NP, neopterin tends to exhibit only pro-oxidant activity during studies of chemiluminescence and bacterial cytotoxicity (Weiss *et al.*, 1993; Murr *et al.*, 1994; Reibnegger *et al.*, 1995; Horejsi *et al.*, 1996; Murr *et al.*, 1996; Wede *et al.*, 1999), as well as nitration (Widner *et al.*, 1998) and LDL oxidation (Herpfer *et al.*, 2002; Greilberger *et al.*, 2004). The neopterin-mediated LDL oxidation occurred whether native, minimally or moderately oxidized LDL was used, but the enhancement was small and dependent on the presence of another oxidizing source (Herpfer *et al.*, 2002; Greilberger *et al.*, 2004). Unlike 7,8-NP, the effects of neopterin were also generally dependent on pH. 3-Nitrotyrosine formation was promoted by neopterin only between pH4 to pH5.5 (Widner *et al.*, 1998), while its enhancement of chloramine T- and hydrogen peroxide-induced chemiluminescence and cytotoxicity required a neutral or slightly alkaline pH (Weiss *et al.*, 1993). Additionally, hydrogen peroxide-induced chemiluminescence was shown to be dependent on the presence of iron chelator complexes (Murr *et al.*, 1994; Murr *et al.*, 1996).

Neopterin's pro-oxidant nature was found to extend to the enhancement of an NADPH-independent release of ROS from adhesive neutrophils (Razumovitch *et al.*, 2003) and to the promotion of UV-A irradiation-induced DNA damage in B-16 melanoma cells (Kojima *et al.*, 1995). Furthermore, apoptosis in the rat alveolar epithelial L2 cell line was increased by neopterin, either alone or in combination with IFN-γ and TNFα (Schobersberger *et al.*, 1996). Neopterin also enhanced apoptosis in rat vascular SMCs (Hoffmann *et al.*, 1998b). By contrast, the presence of up to 1mM

neopterin slightly inhibited TNFα-induced apoptosis in U937 cells (Baier-Bitterlich *et al.*, 1995).

7,8-NP is oxidized to 7,8-dihydroxanthopterin in the presence of oxidants like chloramine T, AAPH, hydrogen peroxide and oxygen (Murr *et al.*, 1996; Oettl *et al.*, 1999; Gieseg *et al.*, 2001a; Duggan *et al.*, 2002). Conversely, co-incubating 7,8-NP with hypochlorite produces neopterin (Widner *et al.*, 2000; Gieseg *et al.*, 2001a). Both modifications contrast with the lack of chemical alteration observed for neopterin upon enhancement of chemiluminescence and this has led to the suggestion that neopterin promotes oxidative damage via an unidentified catalytic mechanism (Murr *et al.*, 1996). Nevertheless, pterin radicals have been detected during neopterin-mediated enhancement of photodamage to DNA (Ito & Kawanishi, 1997). Neopterin's mode of action therefore remains unclear and seems likely to vary between experimental conditions. Under some circumstances neopterin can even be neutral (Oettl & Reibnegger, 2002), as was observed when studying the effect of neopterin on apoptosis in rat PC12 cells (Enzinger *et al.*, 2002b) or human neuronal NT2 cells (Spottl *et al.*, 2000).

1.3.3.4 Gene Expression and Signal Transduction

While disruption of the cellular redox balance promotes apoptosis, it is also suggested to account for the neopterin- and 7,8-NP-mediated activation of various redox-sensitive transcription factors. A two hour incubation with 20μM neopterin enhanced NF-κB translocation to the nucleus of rat vascular SMCs but could be inhibited by pre-incubation with the antioxidant PDTC (Hoffmann *et al.*, 1996). As NF-κB is postulated to participate in iNOS stimulation, it is unsurprising that the same quantity of neopterin has also been observed to upregulate iNOS transcription and nitric oxide release in these cells (Schobersberger *et al.*, 1995; Hoffmann *et al.*, 1996). Another study investigated NF-κB activation in Jurkat T cells transfected with a NF-κB promoter-chloramphenicol acetyltransferase (CAT) reporter gene construct (Baier-Bitterlich *et al.*, 1997). Transcription of this reporter gene construct was significantly upregulated upon the co-incubation of 7,8-NP with TNFα. In the same paper, 7,8-NP alone was shown to enhance transcription of an AP-1-CAT promoter-reporter gene construct. More specifically, the activity of AP-1's *c-fos* subunit has been studied in transfected NIH3T3 fibroblasts (Uberall *et al.*, 1994). 7,8-NP and, to a lesser extent, neopterin were both

able to transactivate a c-*fos*-CAT promoter-reporter gene construct. Further upregulation was observed by co-incubating the pteridines with cyclic guanosine monophosphate (cGMP).

The downstream consequences of pteridine-induced transcription factor activation are not merely restricted to host cells. NF-κB is a known enhancer element of the HIV-1 long terminal repeat (LTR) promoter (Baier-Bitterlich *et al.*, 1997) and, accordingly, a study of Jurkat T cells transiently transfected with an HIV-1-CAT promoter-reporter gene construct has confirmed that 7,8-NP enhances HIV-1 expression (Wirleitner *et al.*, 2005). This pteridine also transactivates the type 1 human T-cell leukaemia virus (HTLV-1) LTR promoter when incubated with Jurkat T cells at a concentration of 5mM (Baier-Bitterlich *et al.*, 1996a).

Even second messengers like calcium appear susceptible to the effects of pteridines, thereby providing an alternative route for neopterin and 7,8-NP to influence cellular signalling. Both compounds, but especially 7,8-NP, were found to elevate intracellular calcium levels in the THP-1 monocyte cell line (Woll *et al.*, 1993). These increases were attributed to the activation of a pteridine-induced calcium channel and, for 7,8-NP, it required as little as 10nM pteridine.

Two cytokines, IFN-γ and TNFα, are also upregulated by pteridines. Incubation with 7,8-NP was reported to transactivate an IFN-γ-CAT promoter-reporter construct in transfected Jurkat T cells (Baier-Bitterlich *et al.*, 1996b). This upregulation was inhibited by NAC and, as a result, is theorized to occur via a ROS-dependent mechanism. Considering IFN-γ's central role in stimulating 7,8-NP synthesis, the 7,8-NP-mediated transactivation also suggests the existence of a positive feed-back loop. Meanwhile, TNFα release was enhanced by neopterin in both human peripheral blood mononuclear cells (Barak & Gruener, 1991) and vascular SMCs (Hoffmann *et al.*, 1998a). Although the former cell type involved co-incubation with IL-2, IFN-γ and LPS, the latter cell type was susceptible to TNFα synthesis in the presence of neopterin alone. Upregulating both these cytokines is expected to promote a pro-inflammatory environment. Such conditions are clearly of significance in atherosclerosis, as is the presence of ICAM-1. It is therefore of relevance that 7,8-NP has been shown to induce ICAM-1 expression in type II-like alveolar epithelial cells (Hoffmann *et al.*, 1999).

Neopterin could potentially contribute to tumour progression, either by preventing nitric oxide-mediated apoptosis or by promoting angiogenesis (Reider *et al.*, 2003). The

former pathway is suggested because ovarian cancer cells are susceptible to nitric oxide and, unlike rat vascular SMCs, neopterin inhibits nitric oxide synthesis in this cell type. Neopterin has also been shown to induce the expression of erythropoietin (EPO) and vascular endothelial growth factor (VEGF) in ovarian cancer cells. Both these proteins are postulated to participate in angiogenesis during tumour development. Neopterin- and 7,8-NP-induced expression of the c-*fos* proto-oncogene provides yet another pathway for pteridine promotion of tumour growth (Reider *et al.*, 2003).

Conversely, 7,8-NP's modulation of the cellular redox balance in Jurkat T cells was found to promote apoptosis by inducing expression of the Fas ligand (Wirleitner *et al.*, 2001). Similarly, 7,8-NP enhanced apoptosis in rat PC12 cells by upregulating signalling cascades associated with mitogen-activated protein kinases (MAPKs) (Enzinger *et al.*, 2001; Enzinger *et al.*, 2002b). Activation of p44/42 ERK was mediated by 7,8-NP alone, while strong activation of SAPK required co-incubation with TNFα.

It is clear that both neopterin and 7,8-NP influence a broad range of cellular functions. In many cases, the exact mechanism(s) of action remain to be elucidated. Nevertheless, a critical and recurring feature appears to be a neopterin- and 7,8-NP-induced disturbance of the cellular redox balance.

1.4 Research Programme

Two major aims define the following research:

- To determine the extent of protein hydroperoxide formation on HMDM cells and LDL exposed to oxidative stress.
- To determine the potential of 7,8-NP to protect HMDM cells and LDL against oxidative stress, whether supplied exogenously or endogenously.

These aims were developed because atherosclerosis results in an environment of oxidative stress. Macrophages and LDL have both been detected in atherosclerotic plaques and are known to be susceptible to oxidative damage. However, to date, most studies have focussed on peroxidation of the lipid components rather than the protein components of these substrates. The role of 7,8-NP *in vivo* remains controversial but, given the inflammatory nature of atherosclerosis, this pteridine is expected to co-exist in atherosclerotic plaque with the cells and LDL. A number of studies have identified an antioxidant activity for 7,8-NP *in vitro*, raising the possibility that macrophages may

synthesize this pteridine to protect themselves and/or other substrates from oxidative damage.

Chapter 3 will therefore describe protein hydroperoxide and lipid hydroperoxide formation in HMDMs exposed to the AAPH peroxyl radical generator. The kinetics of peroxidation will be compared to HMDM viability loss before determining the ability of 7,8-NP to inhibit AAPH-induced oxidative damage and cell death. 7,8-NP will be provided both exogenously and via stimulation of HMDMs with IFN-γ.

The extent of protein hydroperoxide formation during macrophage-mediated LDL oxidation will be described in Chapter 5 and correlated to a range of other LDL oxidation markers. (Chapter 4 will be devoted to resolving the variable rate of cell free LDL oxidation in Ham's F10, thereby enabling accurate conclusions to be drawn in Chapter 5.) Attention will then turn to the efficacy of 7,8-NP in preventing macrophage-mediated oxidation of LDL's protein, lipid and antioxidant components. These experiments will be conducted using both the THP-1 cell line and the more physiologically relevant HMDMs. An attempt will also be made to identify 7,8-NP's mode of action and to confirm whether the IFN-γ-induced effect can be attributed to 7,8-NP or is caused by some other, unspecified, IFN-γ-mediated activity.

The *in vivo* relevance of 7,8-NP will ultimately be described in Chapter 6 by measuring the levels of neopterin and various markers of oxidation in two examples of inflammatory material – pus and atherosclerotic plaque.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Water was deionized and ultrafiltered using a Milli-Q filtration system and, unless otherwise stated, all solutions were prepared in this nanopure water. All reagents used were of analytical grade or better.

α-Naphthyl acetate esterase staining kit	Sigma Chemical Co; St. Louis, MO, USA
α-Tocopherol	Sigma Chemical, Co.
1,1,3,3-Tetramethoxypropane (TMP)	Sigma Chemical Co.
2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH)	Aldrich Chemical Co; Sydney, Australia
2,4-Dinitrophenyl hydrazine (DNPH)	Sigma Chemical Co.
2-Thiobarbituric acid (TBA)	Sigma Chemical Co.
3,4-Dihydroxyphenylalanine (DOPA)	Sigma Chemical Co.
5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB)	Sigma Chemical Co.
7,8-Dihydroneopterin (7,8-NP)	Schircks Laboratory; Switzerland
Ammonium ferrous sulphate	Hopkins & Williams Ltd; Essex, England
Acetic acid	Merck Ltd; Poole, England
Acetone	Merck Ltd
Argon gas	BOC Gasses; NZ
Ascorbic acid	Sigma Chemical Co.
Bathocuproine disulphonic acid (BCS)	Sigma Chemical Co.
Bathophenanthroline disulphonic acid (BPS)	Sigma Chemical Co.
Bicinchoninic acid (BCA) protein determination kit	Pierce; Illinois, USA
Bovine serum albumin (BSA)	Sigma Chemical Co.
Butylated hydroxytoluene (BHT)	Sigma Chemical Co.
Chelex 100 resin	Bio-Rad Laboratories; CA, USA
Chloroform	Merck Ltd and Asia Pacific Speciality Chemicals Ltd; Auckland, NZ
Cholesterol reagent	Roche Diagnostics; USA
Citric acid	BDH Chemicals Ltd; Palmerston North, NZ
Copper chloride	BDH Chemicals Ltd
Cytochrome C	Sigma Chemical Co.
Dialysis tubing	Biolab Scientific; Auckland, NZ
Di-ammonium hydrogen orthophosphate	BDH Chemicals Ltd
Diethyl ether	Merck Ltd
Dimethyl sulphoxide (DMSO)	BDH Chemicals Ltd
Ethanol	BDH Chemicals Ltd
Ethyl acetate	Merck Ltd
Ethylenediaminetetraacetic acid (EDTA)	BDH Chemicals Ltd
Ferricyanide	Sigma Chemical Co.
Formaldehyde	Merck Ltd
Guanidine hydrochloride (GuHCl)	Sigma Chemical Co.

HEPES	Sigma Chemical Co.
n-Hexane	Merck Ltd
Hydrochloric acid (HCl)	BDH Chemicals Ltd
Iodine	BDH Chemicals Ltd
Interferon-γ (IFN-γ)	Imukin®; Boehringer Ingelheim Pty Ltd; NSW, Australia
Iron sulphate	Ajax Chemicals; NSW, Australia
Iron chloride	Sigma Chemical Co.
Lymphoprep	Axis-Shield PoC AS; Oslo, Norway
Neopterin	Schircks Laboratory
Nitrogen gas	BOC Gasses
Mercaptoacetic acid	Sigma Chemical Co.
Methanol	Merck Ltd
3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)	Sigma Chemical Co.
Nitroblue tetrazolium (NBT)	Sigma Chemical Co.
Orthophosphoric acid (85%)	BDH Chemicals Ltd
Perchloric acid (PCA)	BDH Chemicals Ltd
Phenol	Sigma Chemical Co.
Phorbol 12-myristate 13-acetate (PMA)	Sigma Chemical Co.
Potassium bromide	Merck Ltd
Potassium hydroxide	Merck Ltd
Potassium iodide	May & Barker Ltd; Dagenham, England
Sodium acetate	Merck Ltd
Sodium borohydride	Sigma Chemical Co.
Sodium chloride	BDH Chemicals Ltd
Sodium dihydrogen orthophosphate	Merck Ltd
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co.
Sodium hydrogen carbonate ($NaHCO_3$)	Merck Ltd
Sodium hydroxide	Merck Ltd
Sucrose	Chelsea Sugar Refinery; Auckland, NZ
Sulphuric acid	BDH Chemicals Ltd
Superoxide dismutase, bovine erythrocyte (SOD)	Sigma Chemical Co.
Trichloroacetic acid (TCA)	Sigma Chemical Co.
Trifluoroacetic acid (TFA)	Sigma Chemical Co.
Trypan blue solution (0.4%)	Sigma Chemical Co.
Xylenol orange, sodium salt	Sigma Chemical Co.

2.1.2 Media

Earle's balanced salt solution (EBSS)	Sigma Chemical Co.
Ham's F10	Sigma Chemical Co. and Gibco BRL; Auckland, NZ
Hanks' balanced salt solution (HBSS)	Sigma Chemical Co.
Heat-inactivated foetal calf serum (HIFCS)	Gibco BRL
Penicillin/streptomycin (100 units/ml penicillin G and 100μg/ml streptomycin, final concentration)	Gibco BRL
Roswell Park Memorial Institute 1640 (RPMI)	Sigma Chemical Co.
RPMI 1640, without phenol red	Sigma Chemical Co.

2.1.3 General Solutions, Buffers and Media

2.1.3.1 Phosphate Buffered Saline (PBS)

Phosphate buffered saline (150mM sodium chloride and 10mM sodium dihydrogen orthophosphate, pH 7.4) was stirred with 1g of Chelex residue for at least four hours to remove contaminating metal ions before being vacuum filtered through a 0.45µm membrane. If required for cell culture, the PBS was autoclaved (15 minutes, 121°C and 15psi) and then warmed to 37°C immediately prior to use with cells.

2.1.3.2 HPLC Mobile Phases

HPLC mobile phases that contained salt were vacuum filtered through a 0.45µm membrane and degassed via sonication.

2.1.3.3 Cell Culture Media

Powdered medium was dissolved in water, before adding sodium bicarbonate and adjusting the solution to pH 7.4, as per the manufacturer's instructions. Sterilization was accomplished using a peristaltic pump (CP-600, Life Technologies) and a 0.22µm Millex®-GP_{50} filter (Molsheim, SA). Once transferred into sterile bottles, media was stored at 4°C but warmed to 37°C before use.

2.1.3.4 7,8-Dihydroneopterin Solution

A 2mM stock of 7,8-dihydroneopterin (7,8-NP) was prepared fresh prior to each experiment by dissolving in degassed media (Ham's F10 for LDL oxidation experiments; EBSS for AAPH experiments; and RPMI for TPMET experiments) during a five to ten minute sonication. This antioxidant was subsequently sterilized by filtration through a 0.22µm membrane filter.

2.2 Methods

2.2.1 LDL preparation

Ethics approval for the use of human blood was granted by the Canterbury Ethics Committee (protocol number 98/07/069). After an overnight fast, and obtaining written

consent, blood was collected from donors by venipuncture using a 21G x ¾ inch winged infusion set. The blood was drained directly into 50ml Falcon tubes, containing 0.5ml of 10% ($^{w}/_{v}$) EDTA (pH 7.4), and centrifuged in a swing-out rotor at 4°C for 20 minutes at 4,100g with the brake off. The resulting plasma was transferred to centrifuge tubes for a 30 minute spin at 11,000g in a fixed angle rotor to remove any remaining trace of cells. Plasma from all donors was pooled to minimize inter-individual variation and stored in the presence of 0.6% sucrose in 20ml aliquots at -80°C for a maximum of six months (Gieseg & Esterbauer, 1994).

Plasma was thawed, as required, and centrifuged at 4,100g for ten minutes at 4°C to remove any precipitated fibrinogen. The density of the plasma was then adjusted to 1.41g/ml, by dissolving potassium bromide (410mg/ml) in the ice-cold plasma, before being distributed evenly between six Beckman ultracentrifuge tubes.

Three different density gradients of sodium chloride were prepared in a 1mg/ml EDTA (pH 7.4) solution. The densities ranged from 1.08g/ml (solution A) to 1.05g/ml (solution B) and 1g/ml (solution C), with 3ml of each being layered into the untracentrifuge tubes. Solution A was placed immediately above the plasma, followed by solution B and finally solution C (top layer), to establish a discontinuous gradient of decreasing densities. The tubes were then transferred to a Beckman 41-Ti rotor and centrifuged at 10°C and 40,000rpm for 22 hours to ensure isolation of an orange band of LDL in the density range of 1.019g/ml to 1.063g/ml. This band was collected by syringe and, in order to remove all trace of EDTA, was dialysed for 24 hours at 4°C against four changes of degassed PBS containing Chelex 100 resin. Freshly dialysed LDL was stored under argon and filter sterilized through a 0.22µm membrane filter immediately prior to the start of an experiment.

The cholesterol content of LDL was determined by incubating 10µl of LDL with 1ml of cholesterol reagent at room temperature for ten minutes and subsequently measuring the absorbance at 500nm against a cholesterol reagent blank. An LDL concentration was calculated from this absorbance, based on an estimate of cholesterol accounting for 31.64% of the LDL particle by weight and LDL having a molecular weight of 2.5MDa (Gieseg & Esterbauer, 1994).

2.2.2 Cell Culture

A Class II biological safety cabinet (Clyde-Apex BH 200) was used under aseptic conditions for all cell experiments. Sterile plasticware was supplied by Bector Dickinson & Co., Nunc, Nalge Nunc International or Falcon. All other materials were sterilized either by autoclaving (15 minutes, 121°C and 15psi) or by filtration through a 0.22µm membrane filter.

Experiments were conducted on both the THP-1 monocyte-like cell line and primary HMDMs. Cells were stored at 37°C in a humidified incubator, with an atmosphere calibrated to 5% carbon dioxide:95% air (Nuaire™ IR Autoflow).

2.2.3 THP-1 Cell Line

The THP-1 cell line was originally obtained from a patient with acute monocytic leukaemia and has been confirmed to possess monocytic properties (Tsuchiya *et al.*, 1980). Suspensions of the cells were grown in 75cm^2 tissue culture flasks, in RPMI 1640 containing phenol red and L-glutamine and supplemented with 5% ($^v/_v$) heat-inactivated foetal calf serum, 100 units/ml penicillin G and 100µg/ml streptomycin. Cell concentrations were maintained between 2x10^5 cells/ml and 1x10^6 cells/ml by passaging every two to four days. Adverse effects associated with excessive passaging were minimized by removing a fresh stock of THP-1s from liquid nitrogen storage every three to four months.

Trypan blue exclusion staining (Moldeus *et al.*, 1978) provided a quick measure of viability for these suspension cells. Stain was mixed with cell suspension before using a light microscope to count the number of opaque (alive) cells in defined regions of a haemocytometer.

Although THP-1 monocytes were maintained in suspension, all experiments were conducted on THP-1s in their adherent macrophage-like form. Prior to an experiment cells were therefore incubated with PMA, a compound previously shown to promote THP-1 differentiation (Tsuchiya *et al.*, 1982; Graham *et al.*, 1994). Unless otherwise stated, THP-1s were adjusted to a concentration of 1x10^5 cells/ml in RPMI, and PMA (dissolved in DMSO) was added to a final concentration of 100ng/ml. Cells were then plated at 3ml/well in six well adherent plates (for LDL oxidation experiments) or 1ml/well in 12 well adherent plates (for TPMET experiments) and incubated for one week. Fresh medium, supplemented with PMA, was supplied after three to four days.

2.2.4 Human Monocyte-Derived Macrophages

Ethics approval for the use of human blood was granted by the Canterbury Ethics Committee (protocol number 98/07/069). The isolation procedure for human monocyte-derived macrophages (HMDMs) was modified from Christen *et al.* (1994) by Dr Mark Hampton (personal communication) and the Free Radical Biochemistry Laboratory. Once isolated, the cells were maintained in RPMI 1640 containing phenol red and L-glutamine, supplemented with 10% ($^{v}/_{v}$) heat-inactivated human serum, 100 units/ml penicillin G and 100µg/ml streptomycin.

2.2.4.1 Preparation of Human Monocyte-Derived Macrophages

Unlinked blood from consenting haemochromatosis patients was collected into 600ml autologous bags by the NZ Blood Bank (Riccarton Road, Christchurch). On arrival at the Free Radical Biochemistry Laboratory, the blood was aseptically transferred to 50ml Falcon tubes and centrifuged at 3000g, with the brake off, for 30 minutes at room temperature. The resulting buffy coat was isolated and mixed with Hanks' Balanced Salt Solution (HBSS) in a ratio of 15ml:20ml before underlaying this mixture with 15ml of Lymphoprep. (Both solutions had previously been warmed to room temperature.) After centrifuging at room temperature at 1000g for 20 minutes with the brake off, a white layer of monocytes/lymphocytes was visible approximately half-way down the Falcon tube. This layer was transferred to new Falcon tubes and washed four times in 45ml HBSS, with a 15 minute room temperature centrifugation at 500g between each wash step. Cells were subsequently resuspended in serum-free RPMI 1640 (warmed to room temperature), at a concentration of $5x10^6$ cells/ml, and then 7ml per well was aliquoted into six well suspension plates. A small quantity of heat-inactivated human serum (5µl/ml of cell suspension) was added to each well before incubating the plates for approximately 40 hours. Under these conditions, any T cells contaminating the cell suspension are unable to survive while platelets adhere to the surface of the plate. By contrast, monocytes remain both viable and in suspension. After the 40 hour incubation period they were therefore resuspended in fresh RPMI 1640, supplemented with 10% heat-inactivated human serum, at a concentration of either $3x10^6$ cells/ml or $5x10^6$ cells/ml (depending on experimental requirements) and plated at 1ml/well in 12 well adherent plates.

Fresh medium (RPMI 1640 containing 10% heat-inactivated human serum) was supplied every three to four days and experiments were conducted once the majority of monocytes had matured to macrophages, usually 14 days after the initial isolation of the cells. Excess monocytes were greatly reduced in number at the start of an experiment by washing all wells three times in warm, sterile PBS.

2.2.4.2 Preparation of Heat-Inactivated Human Serum

Unlinked blood from consenting haemochromatosis patients was collected into 600ml dry bags by the NZ Blood Bank (Riccarton Road, Christchurch). An overnight incubation, at 4°C, provided time for the serum to completely separate from the blood clot. A sterile mixing canula and syringe were then used to transfer the serum from the bag to 50ml Falcon tubes. The tubes were spun at 400g for 15 minutes to pellet any remaining blood cells and the serum was decanted into new Falcon tubes.

All serum was heat inactivated in a water bath at 56°C for 30 minutes before being cooled and stored at -80°C until required.

2.2.4.3 α-Naphthyl Acetate Esterase Staining Procedure

Cellular esterases are ubiquitous but do exhibit cell-specific differences, thereby providing a convenient means of determining the purity of HMDM preparations. The α-naphthyl acetate esterase enzyme is primarily expressed in monocytes and macrophages and, upon incubation with α-naphthyl acetate, these cells will hydrolyse the ester linkage and release naphthyl. This free naphthyl readily reacts with a diazonium salt, forming a blue-black compound that is clearly visible at the site of the enzyme activity (Moloney *et al.*, 1960).

Before seeding the monocytes at $5x10^6$ cells/ml, sterile glass coverslips (1cm x 1cm) were placed into the wells of 12 well adherent plates. This ensured that the monocytes adhered to, and differentiated directly on, the coverslip. Fourteen days later the coverslips were removed and the cells were fixed by a 30 second immersion in a citrate-acetone-formaldehyde solution (25:65:8), followed by a gentle rinse in water for 60 seconds. The fixed slides were subsequently incubated in the dark for 30 minutes at 37°C in a mixture containing 40ml pre-warmed water, 1ml 0.1M sodium nitrite, 1ml Fast Blue BB Base solution, 5ml TRIZMAL 7.6 buffer concentrate and 1ml α-naphthyl acetate solution (all supplied in the Sigma α-naphthyl acetate staining kit). The slides

were then rinsed thoroughly in water for two minutes before counterstaining in Hematoxylin solution for a further two minutes. Following a final rinse in water, the coverslips were dried and examined microscopically to determine the percentage of cells exhibiting α-naphthyl acetate esterase activity. Cells stained a blue-black colour were considered to be positive for α-naphthyl acetate esterase activity and, consequently, were described as monocytes/macrophages.

2.2.5 AAPH Experimental Set-up

All experiments investigating the effect of AAPH on HMDMs were conducted at a concentration of $5x10^6$ cells/ml and in a total volume of 1ml/well. Cells were washed three times in PBS before the addition to each well of the minimal medium, Earle's Balanced Salt Solution (EBSS), and 0-50mM AAPH. After incubating for the required length of time, HMDM samples were analyzed for cell viability, protein hydroperoxides, lipid hydroperoxides or thiol loss.

When studying the protective capacity of 7,8-NP or IFN-γ, slight modifications to the above protocol were required. 7,8-NP was pre-incubated in EBSS with the HMDMs for ten minutes before initiating the experiment upon addition of AAPH. Experiments investigating IFN-γ required a 48 hour pre-incubation of the HMDMs in fresh RPMI, supplemented with 10% heat-inactivated human serum and up to 1000U/ml of the cytokine. After 48 hours, cells were washed three times in PBS and then incubated in EBSS with appropriate concentrations of IFN-γ and AAPH.

2.2.6 Cell-Mediated LDL Oxidation Experimental Set-up

Ham's F10 has previously been shown to be permissive for cell-mediated LDL oxidation (Graham *et al.*, 1994; Wood & Graham, 1995) and, as such, oxidation experiments using THP-1 macrophage-like cells ($1x10^5$/ml) or HMDMs ($3x10^6$/well) were all conducted in this medium. Some studies required chelexed Ham's F10, which was prepared immediately prior to an experiment by mixing Ham's F10 and Chelex 100 resin (¼ teaspoon per 50ml media) at 4°C for at least four hours. The Chelex was subsequently pelleted by centrifugation and the Ham's F10 was filter sterilized through a 0.22μM membrane filter.

After washing the cells three times in PBS, Ham's F10 (chelexed or non-chelexed) was aliquoted into each well in sufficient quantities to ensure a final volume of 3ml once all reagents were added. Depending on experimental requirements, each well was then supplemented with additional ferrous sulphate, ferric chloride and/or copper chloride and a pre-incubation period of five minutes was allowed before the inclusion of 0.1mg/ml LDL. A control, lacking cells but containing all other reagents, was also prepared. At various time points, the supernatant was removed from the wells and centrifuged at 400g and 4°C for five minutes to pellet any dislodged cells. The resulting LDL supernatant was then analyzed for the level of dienes, thiobarbituric acid reactive substances (TBARS), vitamin E, dityrosine, protein bound 3,4-dihydroxyphenylalanine (PB-DOPA) or protein hydroperoxides.

All 7,8-NP and IFN-γ experiments required additional pre-incubations, with 7,8-NP supplementation occurring five minutes after the addition of the transition metals and requiring an additional five minute pre-incubation before the inclusion of LDL.

Ham's F10 is more nutrient rich than EBSS and therefore, in contrast to the AAPH experiments, it was possible to study the effect of IFN-γ by washing the cells three times in PBS and then pre-incubating 500U/ml of this cytokine for 48 hours in Ham's F10. The incubation was subsequently initiated by removing excess Ham's F10 and replacing it with transition metals, LDL and a small amount of IFN-γ to ensure retention of a final IFN-γ concentration of 500U/ml and a total experimental volume of 3ml.

2.2.7 Cell-Mediated BSA Oxidation Experimental Set-up

Adherent THP-1s ($1x10^5$ cells/ml) were washed in PBS three times before the addition of non-chelexed Ham's F10 to each well and supplementation with 1.5μM ferrous sulphate. A five minute pre-incubation was followed by the inclusion of bovine serum albumin (BSA), with concentrations ranging from 0-5mg/ml. The incubation was halted at various times by removal of the 3ml of supernatant. Dislodged cells were eliminated by centrifugation at 400g for five minutes at 4°C, and the supernatant was then analyzed for protein hydroperoxide formation. A cell free control was included at every time point for comparison.

2.2.8 Pus Preparation

Pus was collected from patients by staff at the Digital Subtraction Angiography (DSA) Unit in Christchurch Hospital's Radiography Department, with ethics approval granted by the Upper South B Regional Ethics Committee (protocol number CTY/0104036) and written consent given by all patients. The pus was subsequently transported to the Free Radical Biochemistry Laboratory on ice and stored at -80°C until required.

After thawing samples under cold running water, 2ml of pus was homogenized in a tissumizer (Tekmar Ltd, USA) with 2ml of water and 50µl each of 20mg/ml BHT (in methanol) and 100mg/ml EDTA (pH 7.4). The total volume was recorded and samples were aliquoted in preparation for the analysis of PB-DOPA, dityrosine, carbonyls, pterin, vitamin E, TBARS and protein content.

2.2.9 Plaque Preparation

The carotid artery was obtained from patients in Christchurch Hospital's Department of Surgery, with ethics approval granted by the Upper South B Regional Ethics Committee (protocol number CTY/0104036). Provided patients had given written consent, it was placed on ice and transported to the Free Radical Biochemistry Laboratory to be weighed and then stored at -80°C.

When ready for analysis, the plaque was thawed and photographed. It was subsequently dissected into sections approximately 3-5mm in length, with the sample furthest away from the bifurcation being labelled as section number one. Transverse views of each fragment were photographed, and weights were also recorded, before homogenizing each section in a tissumizer in the presence of 5ml water and 50µl each of 100mg/ml EDTA (pH 7.4) and 20mg/ml BHT (in methanol). The total volume was noted and samples from each section were then aliquoted for PB-DOPA, dityrosine, carbonyls, pterin, vitamin E, TBARS, protein and cholesterol analysis.

2.2.10 Viability Assays

2.2.10.1 Trypan Blue Exclusion Staining

Trypan blue exclusion staining monitors cell viability via analysis of membrane integrity (Moldeus *et al.*, 1978). Viable cells have intact membranes that are impermeable to the dye and, as a consequence, appear opaque when viewed under a

microscope. By contrast, dead cells possess disrupted membranes and are no longer able to pump the dye out, resulting in a blue appearance.

Cells in suspension can be easily counted on a haemocytometer (section 2.2.3) but adherent cells remain attached to the surface of the plate. Thus, the viability of adherent THP-1s or HMDMs was determined at various time points by removing the incubation medium and submerging the cells in trypan blue. (Approximately 200µl ensured full coverage of the surface area in a 12 well plate.) Interference from all the excess stain was minimized by removal of the dye after 30 seconds and replacement with 1ml of warm PBS. An inverted microscope (40x magnification with a 10x eyepiece), was subsequently used to randomly examine five fields of view across each well. The proportion of viable cells in that well was calculated by dividing the number of viable cells from all five fields of view by the total number of cells (both alive and dead) from all five fields of view.

2.2.10.2 MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay provides an alternative, indirect measure of viability. It is based on the ability of cells to endocytose the yellow MTT compound and subsequently metabolize it, via the action of cellular NAD(P)H dehydrogenases, into a purple MTT-formazan product that can be quantified spectrophotometrically (Mosmann, 1983). As a result, the intensity of the colour provides an indication of both the concentration of cells and their metabolic activity.

After removal of the incubation medium at selected time points, the adherent cells were washed twice in PBS. To each well was added 900µl of non-phenol red RPMI 1640 plus 100µl of 5mg/ml MTT. Samples were then incubated for one to four hours, allowing sufficient time for significant quantities of purple compound to form in the control wells. This range of time is due to the metabolic activity of HMDMs differing from one preparation to the next. Nevertheless, while the incubation period did change between experiments, it was kept constant throughout each individual experiment. All insoluble MTT-formazan crystals were dissolved by mixing 1ml of 10% ($^{w}/_{v}$) SDS (in 0.01M HCl) in each well and incubating for a further 15 minutes. The absorbance was read at 570nm, against a blank lacking cells but containing all other reagents.

2.2.11 Thiol Determination by the DTNB Assay

Thiol concentrations were analyzed using an assay described by Ando & Steiner (1973) and modified by Soszynski *et al.* (1997). It relies on the 1:1 stoichiometry of the reaction between 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and thiols, and the fact that the resulting product is 3-carboxylato-4-nitrothiophenolate (CNTP), a coloured anion with a peak absorbance at 412nm.

2.2.11.1 Total Cellular Thiols

Experiments were halted by washing the wells twice in warm PBS before adding 1ml cold PBS (pH 7.4) and scraping the HMDMs from the surface of the well. Any cells dislodged during the washing steps were collected in 1.8ml microtubes by centrifuging the wash solution for five minutes at 400g. The resulting pellet was mixed with the 1ml scraped cell suspension and all cells were lysed with 0.5ml of 10% ($^{w}/_{v}$) SDS. Following the addition of 15µl of 3mM DTNB (final concentration 30µM), a 30 minute room temperature incubation was conducted in a heated shaking block (Eppendorf Thermomixer 5436). The absorbance was then measured at 412nm, against blanks containing PBS with DTNB and also against cell suspension samples lacking DTNB. Absorbance values were converted into thiol concentrations using an extinction coefficient of 13,600 $M^{-1}.cm^{-1}$ (Boyne & Ellman, 1972).

2.2.11.2 Supernatant Thiols

Supernatant thiols were analyzed during macrophage-like THP-1 transplasma membrane electron transport (TPMET) experiments using a simplified version of the above method. Dislodged cells were removed from the incubation medium by centrifugation at 400g for five minutes. 900µl of the centrifuged HBSS was transferred to a new microtube and incubated with 100µl of 300µM DTNB in a shaking block at room temperature for 30 minutes. The absorbance was then read at 412nm, against both a HBSS blank containing DTNB and a blank containing supernatant with 100µl phosphate buffer (pH 7.4) rather than 100µl DTNB.

2.2.12 FOX Assays

Numerous variations of the FOX assay have been described in the literature, with some designed to detect lipid hydroperoxides (LOOHs) and others more suited to protein hydroperoxide (PrOOH) measurement. The suitability of each assay also depends on the substrate being studied. Despite the differences, all forms of the FOX assay indirectly measure hydroperoxides due to their ability to oxidize ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) (reaction 11). Xylenol orange (XO) subsequently reacts with the ferric ions to form a complex (Fe-XO) that possesses a maximum absorbance at 560nm (reaction 12).

(11) $Fe^{2+} + PrOOH \rightarrow Fe^{3+} + OH^{-} + PrO^{\bullet}$

(12) $Fe^{3+} + XO \rightarrow Fe\text{-}XO$

2.2.12.1 Protein Hydroperoxide Detection on BSA (FOX-1)

The supernatant was removed at selected times from the wells and centrifuged as explained in section 2.2.6. Using a variation of the FOX assay described by Gay *et al.* (1999), the supernatant was then transferred to clean glass tubes and vortexed in the presence of 420µl of 72% ($^{w}/_{v}$) TCA. Protein in the samples was precipitated by storage on ice for ten minutes, with a subsequent centrifugation at 4100g at 4°C for a further 15 minutes. The resulting pellet was resuspended in 1ml of 5% TCA, transferred to 1.8ml microcentrifuge tubes and spun at 10,300g and 4°C for seven minutes. Following a second 5% TCA wash and centrifugation, the dry pellet was resuspended in 900µl of 25mM sulphuric acid (H_2SO_4) and mixed first with 50µl of 5mM xylenol orange (in 25mM H_2SO_4) and then 50µl of 5mM ferrous ammonium sulphate (in 25mM H_2SO_4). Samples were subsequently incubated at room temperature in the dark for 30 minutes before measuring the absorbance at 560nm, against a blank containing 900µl of 25mM sulphuric acid and 50µl each of xylenol orange and ferrous ammonium sulphate. Protein hydroperoxide concentrations were determined using an extinction coefficient of 35,500 $M^{-1}.cm^{-1}$ (Gay *et al.*, 1999).

2.2.12.2 Protein Hydroperoxide Detection on LDL (FOX-2)

The LDL-containing supernatant was removed from cell culture wells at selected times and centrifuged (section 2.2.6) before being analyzed for protein hydroperoxides using a FOX assay protocol described by Gieseg *et al.* (2003). Protein was precipitated during a ten minute incubation on ice with 72% TCA and then centrifuged at 4°C for 15

minutes at 4100g. The resulting pellet was resuspended (still in glass tubes) in 1ml of 1:1 methanol:chloroform and centrifuged for a further 15 minutes. After being air dried, the transparent pellet was dissolved in 900µl of 1:1 acetic acid:water, 50µl of 5mM xylenol orange and 50µl of 5mM ferrous ammonium sulphate. Blanks, lacking LDL but containing all other reagents, were also prepared. A 30 minute incubation in the dark at room temperature was followed by a five minute centrifugation at 4,100g to pellet any debris. The absorbance was recorded at 560nm and an extinction coefficient of 48,000 $M^{-1}.cm^{-1}$ was used to convert absorbance units to protein hydroperoxide concentrations (Gay *et al.*, 1999).

2.2.12.3 Hydroperoxide Detection on HMDMs

HMDMs were analyzed for both lipid and protein hydroperoxides using a method developed by Gay & Gebicki (2003).

Experiments were halted at selected times by removing the supernatant, then washing the adherent cells twice with warm PBS. The PBS and supernatant were combined and centrifuged at 400g for five minutes to pellet any cells dislodged during the incubation and washing steps. Meanwhile, the cells still attached to the bottom of the wells were exposed to 1ml of ice cold water and scraped off the plate. This cell suspension was mixed with the previously pelleted cells, thereby ensuring that all cells were analyzed for either lipid or protein hydroperoxides.

i) Protein Hydroperoxide Detection on HMDMs (FOX-3)

HMDM protein was precipitated in microcentrifuge tubes by incubating 1ml of cell suspension with 90µl of 2M perchloric acid (PCA) on ice for ten minutes. Samples were then centrifuged at 4°C for seven minutes at 10,300g, and the resulting protein pellet was resuspended in 1ml of 0.2M PCA. After centrifuging and discarding the supernatant again, the pellet was dissolved in 1.2ml of 6M guanidine hydrochloride, transferred to glass test tubes and mixed with an equal volume of chloroform (containing 4mM BHT). Phase separation was achieved by centrifuging at 4°C for ten minutes at 4,100g. The upper aqueous layer (950µl) was then transferred to a microtube and washed with 650µl of chloroform (containing 4mM BHT) before the layers were once again separated by centrifugation. This enabled 700µl of the upper aqueous phase to be isolated and subsequently mixed with 40µl of 0.5M PCA, 25µl of 5mM xylenol

orange (in 110mM PCA), 25µl water and 10µl of 5mM ferrous ammonium sulphate (in 110mM PCA). A 60 minute incubation at room temperature, in the dark, was followed by centrifugation for five minutes at 10,300g to pellet any cell debris. Absorbances were measured at 560nm, against controls containing untreated cells, and were ultimately converted to protein hydroperoxide concentrations using an extinction co-efficient of 35,900 $M^{-1}.cm^{-1}$ (Gay & Gebicki, 2003).

ii) Lipid Hydroperoxide Detection on HMDMs (L-FOX)

HMDM lipid was extracted into solvent by vortexing 1ml of cell suspension with 4.5ml of 2:1 ($^{v}/_{v}$) chloroform:methanol (containing 4mM BHT). Samples were briefly stored on ice before separating the phases via centrifugation at 4°C and 4,100g for ten minutes. After drying 2ml of the lower organic phase under argon, the residue was dissolved in 250µl chloroform (containing 4mM BHT) and 460µl methanol (containing 4mM BHT). This solution was transferred to sealable tubes and incubated in the dark at room temperature for 60 minutes with 41µl of 2M PCA, 30µl of 5mM xylenol orange (in 110mM PCA) and 20µl of 5mM ferrous ammonium sulphate (in 110mM PCA). Samples were subsequently centrifuged to pellet any cell debris and absorbances were recorded at 560nm, against a control of untreated cells. An extinction co-efficient of 51,200 $M^{-1}.cm^{-1}$ was used to calculate the concentration of lipid hydroperoxides (Gay & Gebicki, 2003).

2.2.13 TBARS Assay

The TBARS assay provides an alternative means of monitoring lipid peroxidation and is based on a modification of the method described by Draper *et al.* (1993). It relies on the ability of 2-thiobarbituric acid (TBA) and the malondialdehyde (MDA) lipid hydroperoxide break-down product to readily react, with the resulting pink TBA-MDA adduct being fluorometrically detectable via HPLC.

The method of sample collection varied depending on the type of material being evaluated. Plaque and pus analysis required 100µl of plaque homogenate (section 2.2.9) and 100µl of pus supernatant, with the latter being collected after centrifuging previously prepared pus homogenate (section 2.2.8) at 400g for five minutes. HMDM and LDL samples were obtained from cell extracts (section 2.2.12.3) and supernatants (section 2.2.6), respectively.

Once collected, the 100µl samples were mixed with 50µl of 150mM phosphoric acid and any further oxidation was inhibited by the addition of 10µl of 20mg/ml BHT (in methanol). These samples were then stored at -80°C until required for HPLC analysis.

Upon thawing, 50µl of 42mM TBA reagent was added to each tube and a 30 minute incubation was conducted at 95°C in a heated shaking block. Samples were then cooled on ice and all debris was pelleted by centrifuging at 4°C for ten minutes at 10,300g. The HPLC (Shimadzu RF-10AXL, Shimadzu corporation, Japan) injected 20µl of this supernatant through a Phenosphere reverse phase C-18, 4.6 x 150mm, 5µm column (Phenomenex; Auckland, NZ), heated to 30°C. TBARS were fluorometrically detected using excitation and emission wavelengths of 525nm and 550nm, respectively. The mobile phase, consisting of 55% 50mM sodium dihydrogen phosphate buffer (pH 6.8) and 45% methanol, was pumped through the system at a flow rate of 1ml/minute.

TBARS concentrations in all samples were quantified by comparison with the peak areas of 0µM and 1µM MDA standards. Fresh MDA was prepared before each assay by hydrolysis of 1,1,3,3-tetramethoxypropane (TMP) in 2:3 ethanol:water, with subsequent dilution in water to the appropriate concentration. Phosphoric acid and BHT were then added, as per the experimental samples.

2.2.14 Conjugated Diene Assay

Lipid peroxidation can also be monitored by the increase in absorbance at 234nm. This increase has previously been found to correlate with the production of both lipid hydroperoxides and TBARS and is attributed to the formation of conjugated dienes (Esterbauer *et al.*, 1989).

During LDL oxidation experiments the supernatant was removed from the wells and centrifuged, as described in section 2.2.6, before quickly recording the absorbance at 234nm against a blank of Ham's F10 medium only. The sample was then returned to the test tube for subsequent analysis of protein hydroperoxide, vitamin E and TBARS levels. Conjugated diene concentrations were calculated from the absorbance values using an extinction co-efficient of 29,500 $M^{-1}.cm^{-1}$ (Esterbauer *et al.*, 1989).

2.2.15 Vitamin E Assay

Vitamin E levels in pus, plaque and in LDL subjected to cell-mediated oxidation were analyzed using 100µl of pus homogenate (section 2.2.8), plaque homogenate (section 2.2.9) or centrifuged supernatant (section 2.2.6), respectively. Samples were mixed in glass screw-topped test tubes with 400µl water, 10µl of 100mg/ml EDTA (pH 7.4) and 25µl of 20mg/ml BHT (in methanol). Protein precipitation was induced with 500µl of ice cold ethanol and vitamin E was subsequently extracted into 1ml of hexane during a 60 second vortex. Samples were then stored at -80°C and, when required, were thawed and re-vortexed for a further 60 seconds. Phase separation was maximized by centrifugation at 4°C and 1,600g for five minutes before drying 700µl of the upper hexane phase under nitrogen gas. The resulting residue was then dissolved in 50µl cold methanol in preparation for a 20µl injection onto the HPLC (Gieseg *et al.*, 1995).

Vitamin E was detected using excitation and emission wavelengths of 292nm and 335nm, respectively. The mobile phase of 100% methanol was pumped at 1ml/minute, through an Econosphere reverse phase C18, 4 x 125mm, 5µm column (Alltech Associates Inc; USA), warmed to 35°C in the column oven.

A vitamin E stock solution was prepared by diluting approximately 1mg/ml vitamin E in nitrogen-degassed methanol that was overlayed with argon. Using a wavelength of 294nm and an extinction coefficient of 3086 $cm^{-1}.M^{-1}$, the molar concentration could be spectrophotometrically determined (O'Neil *et al.*, 2001). Stock vitamin E was stored at -20°C until required, at which time it was further diluted in methanol to 3µM for use as an HPLC standard.

2.2.16 PB-DOPA and Dityrosine Assay

DOPA and dityrosine are two products of tyrosine oxidation and both were measured in pus (section 2.2.8), plaque (section 2.2.9) and LDL-containing supernatants (section 2.2.6). All samples were collected into glass Durham tubes but pus analysis required 50µl (diluted in 50µl water) of homogenate and plaque analysis required 100µl of homogenate. By contrast, cell-mediated LDL oxidation was halted at various time points and 900µl of the LDL-containing supernatant was collected and centrifuged. Plaque and pus homogenate already contained antioxidants in the homogenate mix (sections 2.2.8 and 2.2.9) but further oxidation in the LDL supernatants had to be prevented by the addition of 10µl each of 20mg/ml BHT (in methanol) and 100mg/ml

EDTA. LDL samples were subsequently mixed with 140µl of 72% TCA, while plaque and pus were mixed with 900µl of ice cold acetone. All were stored on ice for ten minutes, followed by a 15 minute centrifugation at 4°C and 7000g. The resulting protein pellet was washed in 500µl of -20°C diethyl ether and centrifuged as before. Once drained of all residual diethyl ether, the pellets were stored at -80°C until required or subjected to acid hydrolysis immediately (Gieseg *et al.*, 2003).

In preparation for acid hydrolysis, the Durham tubes were placed into Pico-Tag reaction vials (Millipore, USA) that contained 1ml of 6M hydrochloric acid with 1% ($^{w}/_{v}$) phenol and 50µl mercaptoacetic acid (Gieseg *et al.*, 1993). Air was removed by flushing with argon gas for ten minutes and then evacuating each vial using a vacuum pump. Acid hydrolysis was initiated by incubating at 110°C for 16 hours and the resulting hydrolysis residue was then dried in a speed vac. Once dry, it was dissolved in either 200µl (for plaque and pus) or 50µl (for LDL) of 0.1% TFA and the suspensions were centrifuged to pellet any excess material.

Ultimately, 10µl was injected through a Phenomenex reverse phase C18, 250 x 4.6mm, 5µm column (heated to 35°C), with fluorometric detection using an excitation wavelength of 280nm and an initial emission wavelength of 320nm. After 13 minutes, the emission wavelength was increased to 410nm for dityrosine detection. The 0.1% TFA (pH 2.5) mobile phase was run at 1ml/minute in an acetonitrile gradient, beginning at 0% but rising to 5% after ten minutes; 10% after 14 minutes; and 50% after 16 minutes. By 23 minutes, the acetonitrile concentration was reduced to 1% and this dropped to 0% at the end of the run (30 minutes).

DOPA and tyrosine standards were prepared fresh, dissolving the powder in water via sonication, and then diluting to an appropriate concentration in 0.1% TFA. A dityrosine stock had previously been prepared in the Free Radical Biochemistry Laboratory by a technique involving horseradish peroxidase-mediated oxidation of tyrosine and subsequent purification on DEAE-sephacel (Sigma). The concentration of this dityrosine stock was determined spectrophotometrically at 315nm, using an extinction co-efficient of 5080 $cm^{-1}.M^{-1}$ (Pichorner *et al.*, 1995). It was then stored at -20°C and, when required, aliquots were thawed and diluted to 1µM in 0.1% TFA.

2.2.17 Cholesterol Determination

Cholesterol levels in plaque homogenate were measured as described for LDL cholesterol (section 2.2.1). However, it was important to first dilute the plaque homogenate to ensure that the colour was fully developed after a 30 minute incubation. (This contrasts with the 10 minute period required for LDL). Once colour development was complete, the absorbance was recorded at 500nm against a cholesterol reagent blank.

2.2.18 Carbonyl Assay

Protein hydroperoxide levels in plaque and pus could not be accurately measured using the FOX assay due to the tendency of hydroperoxides to degrade. Carbonyls also serve as a general marker of protein oxidation and, in contrast to hydroperoxides, are significantly more stable. Furthermore, a good correlation between protein hydroperoxide and carbonyl formation has been demonstrated during AAPH-induced oxidation of plasma (Ling, 2003). A protocol described by Quinlan *et al.* (1994) was therefore used to detect carbonyls in plaque and pus homogenate and during cell-mediated LDL oxidation studies. It relies on the ability of carbonyls to react with 2,4-dinitrophenyl hydrazine (DNPH), forming a carbonyl-DNPH derivative with maximum absorbance at 360nm.

Homogenate (200µl) was mixed in screw-topped glass tubes with either 1ml of 10mM DNPH (prepared in 2M HCl) or, in the case of blanks, with 1ml of 2M HCl. All samples (blanks included) were shaken gently at 37°C for 90 minutes before being cooled on ice in the presence of 1ml of 28% TCA. A ten minute centrifugation at 4,100g and 4°C pelleted the precipitated protein, which was then washed twice in 5ml of 1:1 ethanol:ethyl acetate. Once dry, the pellets were resuspended in 1ml of 6M guanidine hydrochloride (in 2M HCl) and colour was allowed to develop during a one hour darkened incubation at room temperature. Excess material was subsequently removed by a five minute centrifugation at 4,100g and absorbances were recorded at 360nm. The HCl blank absorbances were subtracted from the corresponding DNPH treatments before calculating carbonyl concentrations using an extinction co-efficient of 21,000 $cm^{-1}.M^{-1}$ (Quinlan *et al.*, 1994)

Carbonyls on LDL were similarly detected, though protein in the centrifuged supernatant (section 2.2.6) was first precipitated by a ten minute incubation on ice with

72% TCA and then a 15 minute centrifuge at 4°C and 5000rpm. The resulting pellet was washed once in 5% TCA before being dissolved in 1ml of either 10mM DNPH (in 2ml HCl) or 2M HCl and subsequently treated as described for homogenate samples.

2.2.19 Protein Determination

Protein levels were investigated in pus homogenate (section 2.2.8), plaque homogenate (section 2.2.9) and cell suspensions. These cell suspensions consisted of either THP-1s or HMDMs and were prepared as described in section 2.2.12.3, but using only 0.5ml of cold water.

Protein analysis was conducted using a bicinchoninic acid (BCA) protein determination kit from Sigma, with working reagent freshly prepared by mixing Reagent A (sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1M sodium hydroxide) and Reagent B (4% hydrated copper sulphate) in a ratio of 50:1. Thus, once samples were suitably diluted, 50µl was mixed with 1ml of working reagent and incubated at 60°C for 30 minutes in a heated shaking block. The reaction was subsequently halted by placing samples on ice and absorbances were recorded at 562nm against a water blank incubated with the working reagent. Protein concentration was quantitatively determined from a standard curve prepared by the incubation of known concentrations of BSA (0-250µg/ml) in 1ml of working reagent.

2.2.20 Pterin Assay

Neopterin and total pterin (neopterin plus 7,8-NP) levels were measured in pus homogenate (section 2.2.8) and plaque homogenate (section 2.2.9), as well as during studies investigating the IFN-γ-induced production of 7,8-NP in both macrophage-like THP-1s and HMDMs. These IFN-γ experiments monitored pterin levels in the cell-conditioned medium, and in the cells themselves, by collecting centrifuged supernatant (section 2.2.6) and by washing cells in PBS before lysing them in 0.5ml of ice cold water (section 2.2.12.3).

Irrespective of the method of sample collection, a 200µl sample was ultimately aliquoted into microcentrifuge tubes and mixed with 20µl of 50% TCA (Gieseg *et al.*, 2000b). When measuring total pterin, the oxidation of all 7,8-NP to neopterin was ensured by the additional inclusion of 16µl of an acidic iodide solution (5.4% $^{w}/_{v}$ iodine

and 10.8% $^{w}/_{v}$ potassium iodide in 3.7% $^{w}/_{v}$ TCA). After incubating in the dark for 60 minutes, a few crystals of ascorbate were added to remove excess iodine. All samples (whether measuring total pterin or just neopterin) were centrifuged at 4°C and 10,300g for ten minutes, with 100µl of the resulting supernatant then being mixed with 5µl of 2M ammonium phosphate (pH 6). The HPLC injected 20µl of this mixture through a reverse phase Develosil C18, 250 x 4.6mm, 5µm column (Nomura Chemicals; Japan), heated to 35°C. The mobile phase consisted of 20mM ammonium phosphate (pH 6) with 5% methanol and was pumped at 1ml/minute. Neopterin was fluorometrically detected using excitation and emission wavelengths of 353nm and 438nm, respectively. When present in sufficient quantities, 7,8-NP was directly monitored via the HPLC electrochemical detector (ECD) using a voltage setting of +0.6V.

A stock of neopterin was prepared by sonication in 10mM phosphoric acid and stored at -20°C. When required, it was thawed and diluted to a 1µM standard in 10mM phosphoric acid. By contrast, the 7,8-NP standard was prepared by sonication in degassed water and then diluted in 10mM phosphoric acid for immediate use.

2.2.21 Transplasma Membrane Redox Activity Assays

Transplasma membrane electron transport (TPMET) systems are expressed in every living cell (Ly & Lawen, 2003) and can be monitored via assays that measure the degree of transition metal reduction induced by both the cells and the cell-conditioned medium. The latter activity is attributed to reductants released from cells while the difference between the two accounts for TPMET activity. Two such assays were used in this study to monitor the TPMET activity of macrophage-like THP-1s. They are based on a protocol described by Baoutina *et al.* (2001b) and measure the reduction of cupric ions and ferricyanide to cuprous ions and ferrocyanide, respectively.

2.2.21.1 TPMET Reduction of Copper

THP-1 macrophages were washed three times in PBS and then incubated at 37°C with 900µl HBSS in the presence or absence of 50µl each of 1mM copper chloride and 2.5mM bathocuproine disulphonic acid (BCS). After two hours, the medium was removed and any dislodged cells were eliminated by centrifuging at 400g for five minutes. The absorbance of medium incubated in the presence of copper chloride and BCS was read immediately at 482nm, against controls incubated in the absence

of cells. Cuprous ion concentrations were quantified using an extinction co-efficient of 12,200 $cm^{-1}.M^{-1}$ (Baoutina *et al.*, 2001b).

Medium incubated in the absence of copper chloride and BCS served as the cell-conditioned medium and, once centrifuged, cell-conditioned medium was therefore transferred to cell-free wells and incubated with copper chloride and BCS for a further two hours. Absorbances were then measured at 482nm, against a control incubated in the absence of cells.

2.2.21.2 TPMET Reduction of Ferricyanide

After THP-1 macrophages were washed three times in PBS, these cells were incubated in 900µl HBSS at 37°C for two hours in the presence or absence of 100µl of 1mM potassium ferricyanide. The medium was subsequently centrifuged at 400g for five minutes and 0.5ml of the supernatant mixture was then combined with 0.1ml each of 3M sodium acetate (pH 6.5), 0.2M citric acid, 3.3mM ferric chloride (in 0.1M acetic acid) and 3.3% ($^{w}/_{v}$) bathophenanthroline disulphonic acid (BPS). A ten minute incubation at room temperature in the dark provided time for colour development before recording the absorbance at 535nm, against controls incubated in the absence of cells but containing all other reagents. Absorbances were converted to ferrocyanide concentrations using an extinction co-efficient of 21,600 $cm^{-1}.M^{-1}$ (Baoutina *et al.*, 2001b).

The centrifuged cell-conditioned medium was ultimately transferred to cell-free wells and incubated with potassium ferricyanide for a further two hours before 0.5ml was treated with 0.1ml each of the reagents listed above, incubated for ten minutes and read at 535nm.

2.2.21.3 TPMET Experiments Studying the Effect of 7,8-NP, IFN-γ and Superoxide

7,8-NP interferes with both TPMET assays and, as such, it was necessary to modify the above protocols by including a pre-incubation step during 7,8-NP studies. Pre-incubation involved maintaining the cells in RPMI at 37°C, but in the presence of 50µM 7,8-NP for two or six hours. The TPMET assays were subsequently initiated by washing all wells three times in PBS and then monitoring either ferricyanide or cupric ion reduction.

Unlike 7,8-NP, IFN-γ doesn't interfere with either TPMET assay. Thus, after pre-incubating cells in RPMI with 500U/ml IFN-γ for 24 or 48 hours, fresh IFN-γ (diluted in HBSS) was added at the start of the TPMET assay to replace that lost during the PBS washing steps.

The effect of superoxide on ferricyanide and cupric ion reduction was also directly measured. TPMET experiments were set up as above, but the two hour incubation was conducted in the presence and absence of 40µg/ml superoxide dismutase (SOD).

2.2.22 Reducing Activity Associated with Cell-Conditioned Medium

Reducing activity detected in the cell-conditioned medium during the ferricyanide and cupric ion reduction assays has been suggested to be due to reductants released from cells, particularly thiols and superoxide (Garner *et al.*, 1997a; Baoutina *et al.*, 2001b). Supernantant thiols were measured as previously described (section 2.2.11b), while superoxide production was investigated via both the cytochrome C and nitroblue tetrazolium (NBT) reduction assays.

2.2.22.1 Superoxide Measurement via Cytochrome C Reduction Assay

The ability of THP-1 macrophages to reduce cytochrome C was measured using an assay described by Jessup *et al.* (1993). Cells were washed three times in PBS before being incubated for two hours in a 1ml solution, containing HBSS and 80µM cytochrome C in the presence or absence of 40µg/ml SOD. The supernatant was then centrifuged for five minutes at 400g before measuring the absorbance at 550nm, against a cell free control. Concentrations of reduced cytochrome C were determined using an extinction co-efficient of 21,000 $cm^{-1}.M^{-1}$ (Niu *et al.*, 2000), with the difference between the level of reduced cytochrome C in the presence and absence of SOD being attributed to superoxide.

2.2.22.2 Superoxide Measurement via NBT Reduction Assay

A protocol based on the Rook *et al.* (1985) method was used to monitor THP-1-macrophage-mediated reduction of NBT. This required washing the cells three times in PBS, followed by a two hour incubation in a 1ml solution of HBSS with 1mg/ml NBT in the presence or absence of 40µg/ml SOD. All wells were subsequently washed three times in 70% methanol, both to fix the cells and remove any unreduced NBT, before air

drying the wells and dissolving the insoluble formazan in 450µl of 2M potassium hydroxide and 525µl DMSO. The absorbances of the resulting solutions were read at 630nm, against identically treated cell free controls, and the concentrations of reduced NBT were calculated using a standard curve that had been prepared by dissolving known concentrations of NBT (0-20nM) in an 18:21 ratio of 2M potassium hydroxide:DMSO. Superoxide levels were calculated as the difference in reduced NBT between samples incubated in the presence and absence of SOD.

2.2.23 Statistical Analysis

Data was graphed and statistically analyzed using the software program, Prism (version 4.0; GraphPad Software, USA). Significance was confirmed by either a one-way analysis of variance (ANOVA) or a multiple analysis of variance (MANOVA), followed by Tukey's multiple comparison test to provide a more quantitative indication of significance between treatments and/or time points. Where appropriate, linear regression and correlation analyses were also applied, with r^2 values calculated assuming a Gaussian distribution. Significance levels are indicated in the following manner: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Results displayed in this thesis are taken from one experiment, which is representative of three separate experiments. The means and standard errors of the mean (SEM) shown within each experiment were calculated from triplicate samples.

3 7,8-NP and AAPH-Induced Damage to HMDM Cells

3.1 Introduction

AAPH has been used to induce oxidative stress in a variety of experimental systems, ranging from pure protein solutions to lipid micelle suspensions, lipoproteins, plasma, erythrocyte membranes and even living cells (Lissi *et al.*, 1991; Gieseg *et al.*, 1995; Sato *et al.*, 1995; Nourooz-Zadeh *et al.*, 1996; May *et al.*, 1998; Ma *et al.*, 1999; Gieseg *et al.*, 2000b; Park *et al.*, 2002; Gieseg *et al.*, 2003; Yoshida *et al.*, 2004). It has also been utilized *in vivo* as a source of free radicals during animal studies (Dooley *et al.*, 1990; Niki, 1990). While AAPH is not a physiologically relevant compound, it does decompose to two carbon-centred radicals (reaction 13) which each react almost instantaneously with oxygen to produce peroxyl radicals (reaction 14).

(13) $R\text{-}N{=}N\text{-}R \xrightarrow{37°C} N_2 + 2R^{\bullet}$

(14) $2R^{\bullet} + O_2 \longrightarrow 2ROO^{\bullet}$

This allows AAPH to serve as an appropriate model of oxidative stress because peroxyl radicals are known to form *in vivo*, particularly during peroxidation reactions. Moreover, the rate and extent of radical generation can be carefully controlled when using AAPH, greatly aiding kinetic analysis. The rate of decomposition for this water-soluble azo compound depends predominantly on temperature, with solvent and pH playing only a minor role (Niki, 1990). In addition, the half-life for AAPH decomposition is 175 hours, ensuring that the rate of radical generation is constant for the first several hours (Niki, 1990). Thus, at 37°C and in the presence of oxygen, the rate of radical generation is determined by the following equation:

$$\text{Rate} = 3.19\text{x}10^{-7} \text{ x } [\text{AAPH}]$$

where [AAPH] is the molar concentration of AAPH and the units for rate are molar per second (Yamamoto *et al.*, 1985).

While AAPH-induced damage has been studied in both the U937 and THP-1 monocytic cell lines (Duggan *et al.*, 2002; Baird *et al.*, 2005; Kappler, 2005), there is a distinct lack of research on AAPH-induced damage to HMDMs. This paucity of knowledge is largely due to the fact that primary cells are more difficult and time

consuming to work with than cell lines. Donor variability and finite life-time are other negative aspects associated with primary cells. However, by their very nature, cell lines are not completely representative of the corresponding cells in the body. The effect of this on experimental results has recently been highlighted with the discovery that oxLDL-induced cell death occurs by different mechanisms in U937 and THP-1 cells (Baird *et al.*, 2004). While both cell lines undergo apoptosis, THP-1s do so via activation of the caspase enzymes but U937s enter a less conventional apoptotic pathway. Of more relevance to the current research, it has also been found that 7,8-NP's ability to protect monocytes against AAPH-induced damage varies depending on the cell line (Baird *et al.*, 2005). 7,8-NP significantly inhibited thiol loss and cell death in U937s (Duggan *et al.*, 2002; Baird *et al.*, 2005) but had no effect on thiol levels and only a limited impact on viability in THP-1s (Baird *et al.*, 2005). This disparity makes it very difficult to determine which cell line provides the most accurate model of cells *in vivo*. Primary cells are generally considered more physiologically relevant than cell lines and, as such, this section of research will extend the findings of Baird *et al.* (2005) by investigating the influence of AAPH and 7,8-NP on viability and total cellular thiol loss in HMDMs.

The effect of AAPH and 7,8-NP on HMDM hydroperoxide formation will also be studied. Protein rather than lipid is the most significant site of AAPH-induced hydroperoxide generation on both U937s (Gieseg *et al.*, 2000a) and red blood cell membranes (Firth, 2001). Furthermore, protein hydroperoxides have been detected on THP-1s at levels comparable to those measured in U937s (Cassidy, 2003; Kappler, 2005). The current research will therefore determine whether a similar trend is obtained upon incubation of AAPH with HMDMs. It will also investigate the effectiveness of 7,8-NP in preventing HMDM hydroperoxide formation because this antioxidant has previously been found to inhibit the generation of protein hydroperoxides on U937s exposed to AAPH (Duggan *et al.*, 2002).

3.2 Results

3.2.1 Concentration-Dependent Effect of AAPH on Cellular Viability

Before beginning time course and 7,8-NP studies, a suitable concentration of AAPH first needed to be identified by incubating 14 day cultures of HMDMs ($5x10^6$ cells/ml) in EBSS for 12 hours in the presence of a range of AAPH concentrations. Loss of

viability was measured by MTT and trypan blue exclusion staining and exhibited a clear dependence on AAPH concentration, with r^2 values of 0.90 and 0.99, respectively (Figure 3.1). For ease of comparison, results from both assays were displayed as percentages of the control. Nevertheless, it should be noted that cells in control treatments consistently exceeded 98% viability prior to any data manipulation, as determined by trypan blue exclusion staining (data not shown).

Subsequent experiments were conducted using 20mM AAPH because this concentration reduced viability to approximately 50% (44.4 ± 3.4% and 58.0 ± 1.4% by MTT and trypan blue assays, respectively). This result was reproducible, though inter-experimental variation did account for a few exceptions when viability loss was as little as 65% or, conversely, as low as 40% (data not shown).

The discovery of a 50% HMDM mortality rate being reached after 12 hours in the presence of 20mM AAPH was unexpected because a previous study had indicated that 10mM AAPH was sufficient to produce such an effect (Duggan, 2000). This disparity may simply be due to inter-experimental variation or, alternatively, the cells used in the Duggan study may have been less differentiated. To resolve this issue, the above experiment was repeated but three day rather than 14 day cultures were tested (Figure 3.2). A concentration-dependent effect of AAPH on cell viability was once again observed. However, in contrast to Figure 3.1, cell death reached approximately 50% with only 10mM AAPH (59.0 ± 5.1% and 52.1 ± 5.1% by MTT and trypan blue assays, respectively). By 20mM AAPH, the percentage of dead cells had climbed to almost 90%.

All subsequent experiments investigated the effects of AAPH and 7,8-NP using the more differentiated 14 day HMDM cultures.

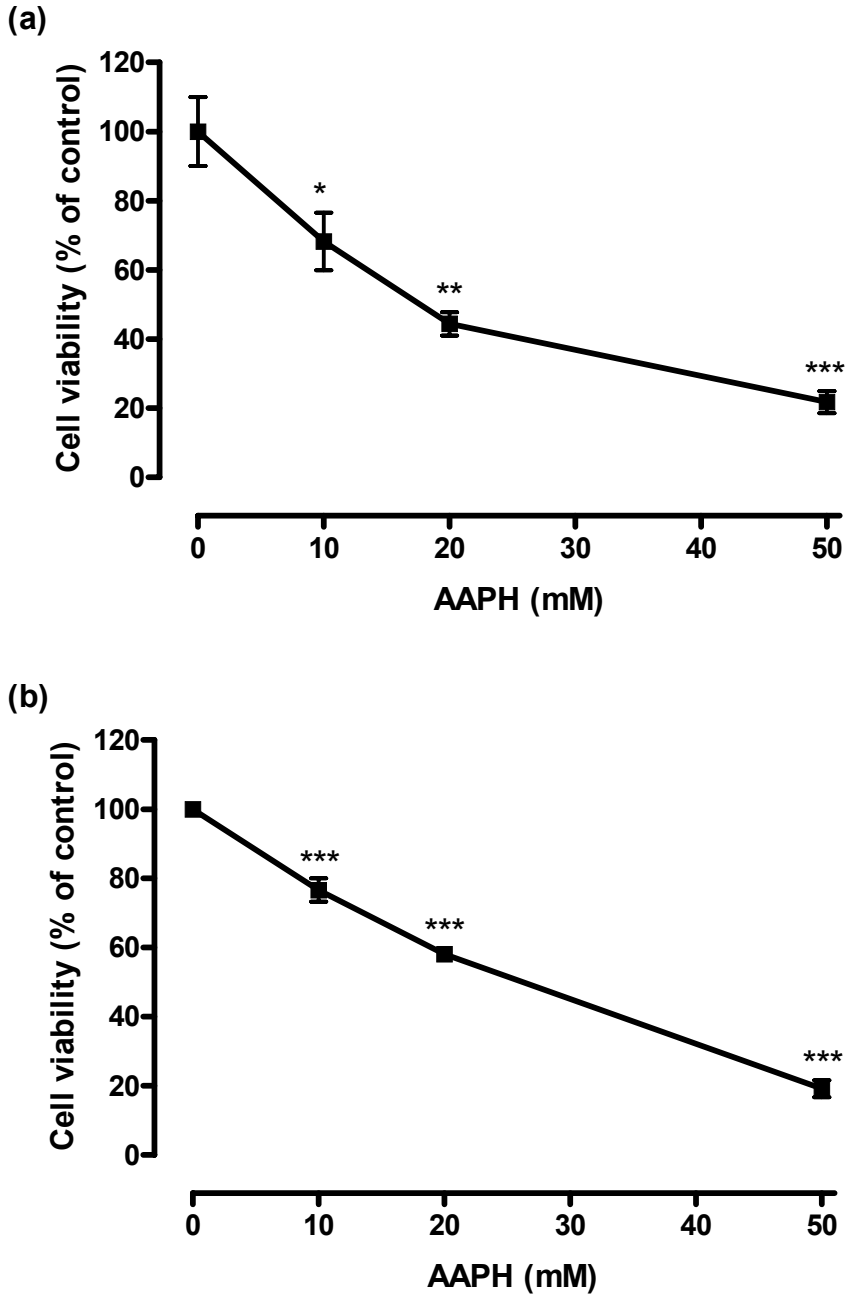


Figure 3.1 Loss of HMDM viability in 14 day cultures with increasing concentrations of AAPH.

14 day cultures of HMDMs ($5x10^6$ cells/ml) were incubated at 37°C in EBSS with increasing concentrations of AAPH. After 12 hours, HMDMs were analyzed for cell viability via (a) MTT and (b) trypan blue exclusion staining. Significance is indicated from 0mM AAPH. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

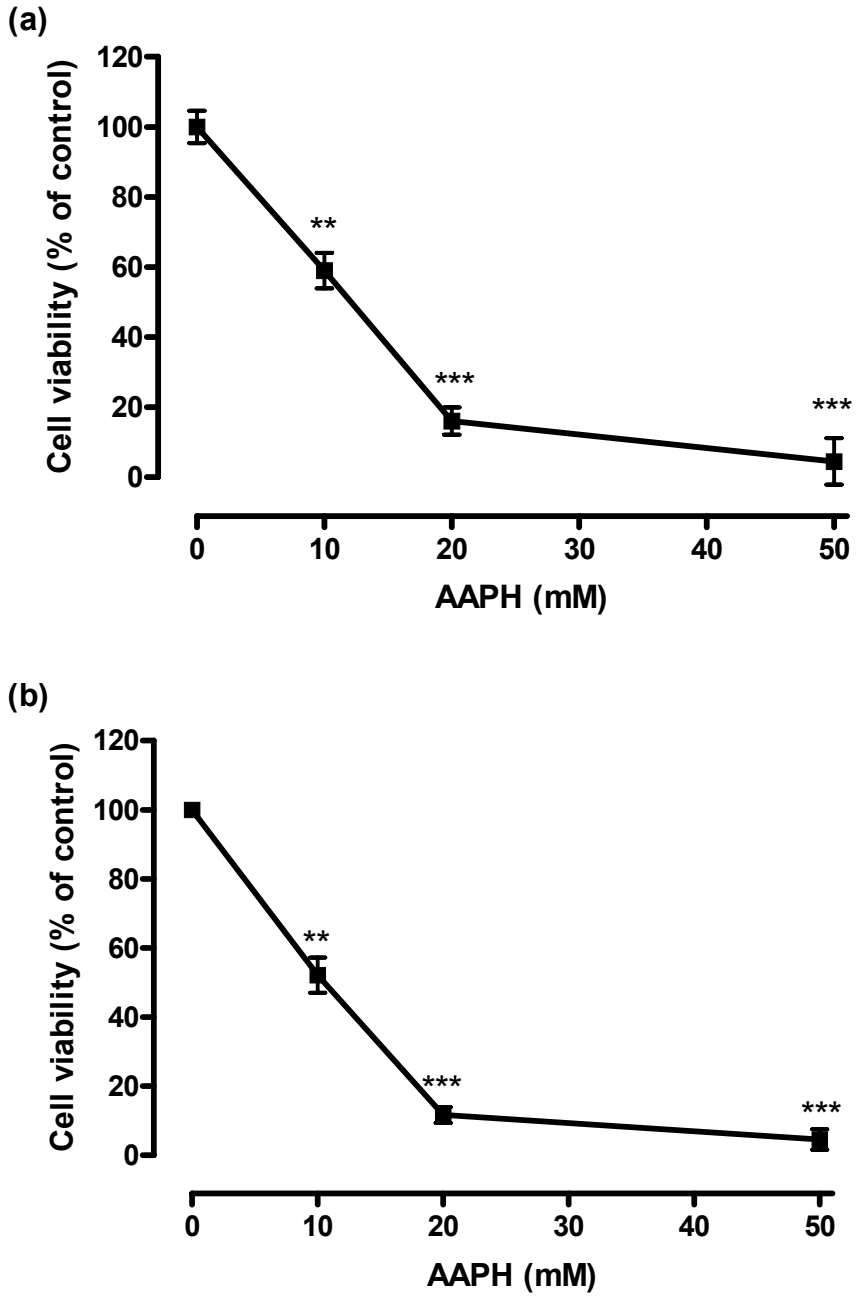


Figure 3.2 Loss of HMDM viability in three day cultures with increasing concentrations of AAPH.

Three day cultures of HMDMs ($5x10^6$ cells/ml) were incubated at 37°C in EBSS with increasing concentrations of AAPH. After 12 hours, the human monocytes were analyzed for cell viability via (a) MTT and (b) trypan blue exclusion staining. Significance is indicated from 0mM AAPH. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

3.2.2 AAPH-Induced Hydroperoxide Formation on HMDMs

The FOX-1 assay lacks a lipid extraction step but has been successfully used to measure protein hydroperoxides in U937s due to the absence of significant quantities of lipid hydroperoxides forming in this cell line after AAPH treatment (Gieseg *et al.*, 2000a). Whether the FOX-1 assay would also accurately detect protein hydroperoxides was unclear and needed to be tested. Thus, hydroperoxides were generated on HMDMs by exposure to 20mM AAPH for 16 hours. Lipids were then completely eliminated from 72% TCA-precipitated pellets, using either an acetone followed by a diethyl ether extraction (Figure 3.3a) or a methanol then hexane extraction (Figure 3.3b), and samples were analyzed by the FOX-1 assay. Some variability in hydroperoxide concentrations is apparent between the two experiments but this is most likely due to donor variability and does not detract from the fact that both lipid extraction procedures reduced hydroperoxide levels. The acetone/diethyl ether extraction reduced hydroperoxides to the extent that, while the level was not significantly different from the 5% TCA washed sample, it was also not significantly different from the baseline value recorded in the absence of any AAPH (Figure 3.3a). Hydroperoxide loss was even more pronounced using the methanol/hexane wash (Figure 3.3b), with levels dropping considerably below the TCA treatment ($p<0.001$). As any hydroperoxides still detectable after lipid extraction are associated with protein, these results imply that protein hydroperoxides were present at levels of only 0.28 ± 0.13μM and 0.39 ± 0.16μM after lipid extraction by acetone/diethyl ether and methanol/hexane, respectively. A similarly low level of 0.27 ± 0.02μM protein hydroperoxides was detected after extracting lipids using a methanol/chloroform wash (data not shown). Including a 5% TCA wash step, subsequent to lipid extraction with solvents, also failed to prevent the sudden drop in hydroperoxides (data not shown).

Protein hydroperoxide formation on HMDMs is clearly limited, suggesting that lipid rather than protein may be the primary target of peroxyl radical attack on this cell type. TBARS provides a measure of lipid peroxidation and, as such, was used to investigate this theory. However, while TBARS levels did increase significantly by 12 hours in the presence of 20mM AAPH ($p<0.001$), the concentration remained low and plateaued at 106.9 ± 3.1nM (equivalent to 21.4 pmole/million cells) after 18 hours (Figure 3.4).

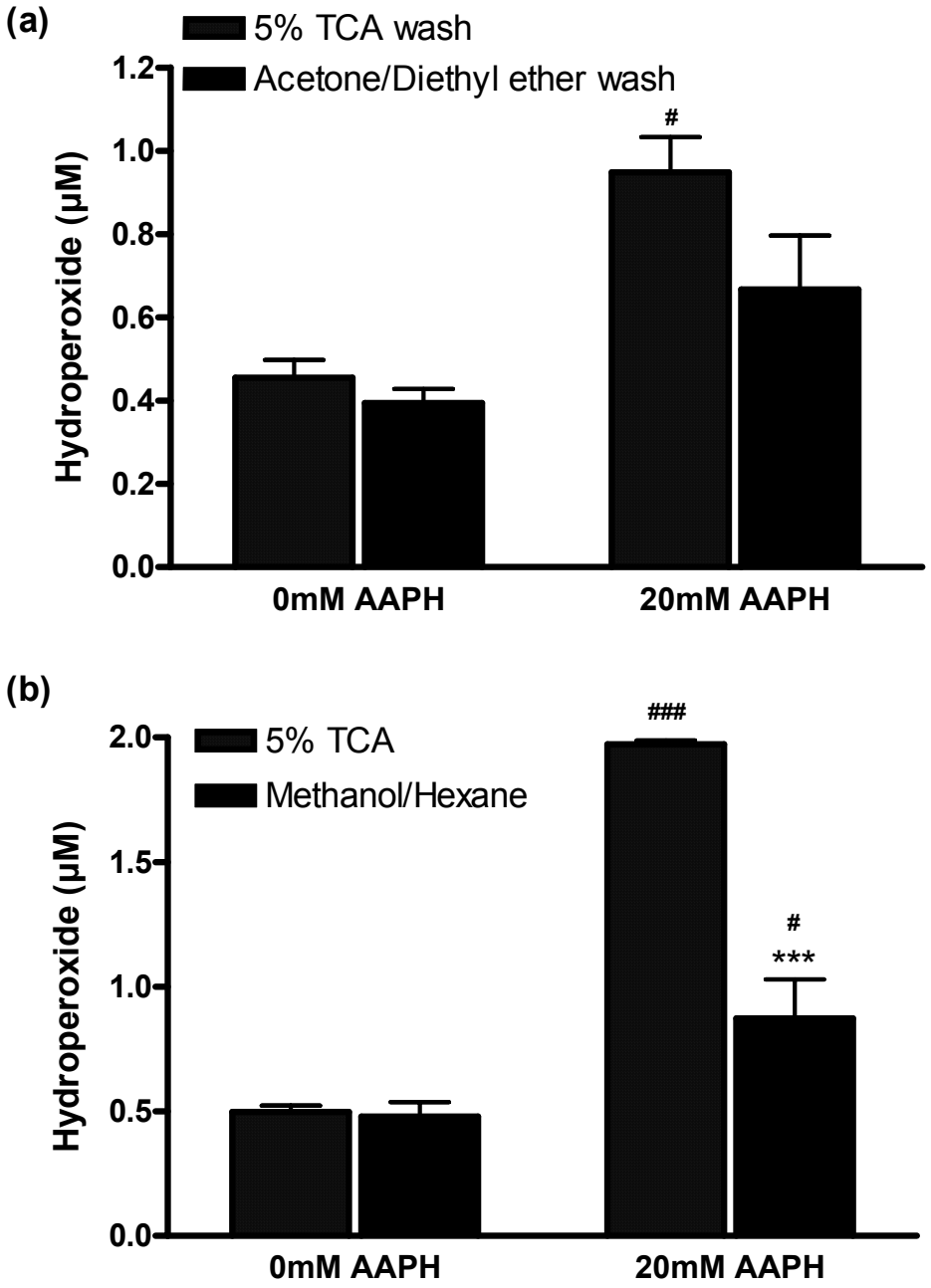


Figure 3.3 Effect of lipid extraction from HMDMs on hydroperoxide detection. HMDMs ($5x10^6$ cells/ml) were incubated at 37°C for 16 hours in EBSS in the presence or absence of 20mM AAPH. Hydroperoxides were measured in half the samples by the FOX-1 assay (with 5% TCA washes) while hydroperoxides in the remaining samples were measured using a modification of the FOX-1 assay, with washes of the 72% TCA-precipitated samples in either (a) acetone followed by diethyl ether or (b) methanol then hexane. The micromolar values refer to concentrations in the original cell suspension. * indicates significance between the wash treatments. # indicates significance from 0mM AAPH. Results are displayed as mean ± SEM of triplicates from a single experiment, with (a) and (b) representing different donors.

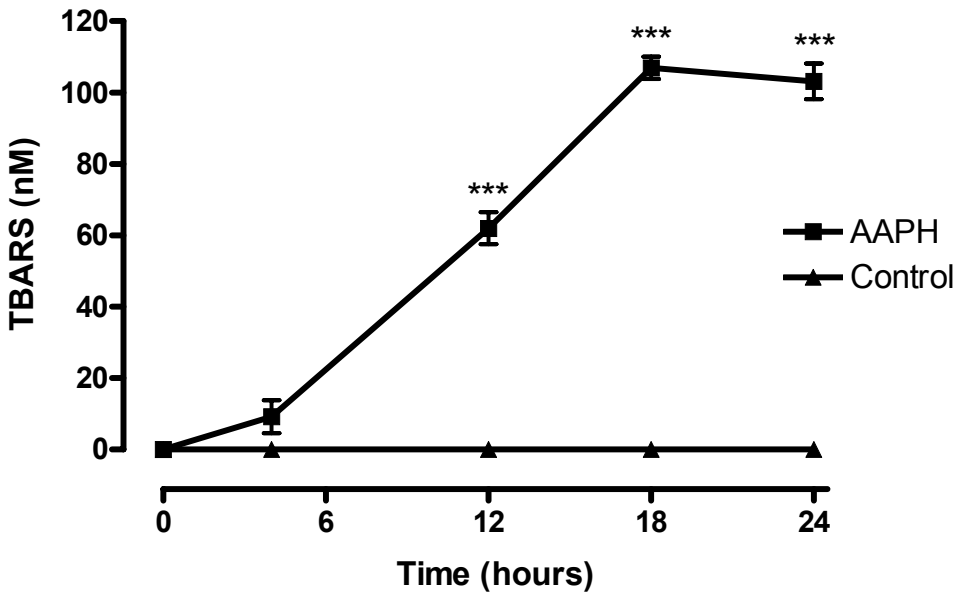


Figure 3.4 AAPH-induced TBARS formation on HMDMs.
HMDMs ($5x10^6$ cells/ml) were incubated at 37°C in EBSS in the presence or absence (control) of 20mM AAPH. Samples were removed at various time points and analyzed for the level of TBARS associated with HMDMs. The nanomolar value refers to the concentration in the original cell suspension. Significance is indicated from time zero. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

A more sensitive and direct method for detecting lipid hydroperoxides was reported soon afterwards by Gay & Gebicki (2003) and is derived from previous lipid FOX assays. This most recent L-FOX protocol has the added advantage of being convenient for use in parallel with the FOX-3 assay when measuring lipid and protein hydroperoxides from the same experiment. HMDMs exposed to 20mM AAPH for 16 hours were therefore analyzed for both lipid and protein hydroperoxides by the L-FOX and FOX-3 assays, respectively. A general measure of hydroperoxides provided a further comparison to the FOX-1 assay results in Figure 3.3 and was obtained by using a modified version of the FOX-3 assay, consisting of two PCA washes and no chloroform washes. Blanks were included in each assay and comprised reagents in the absence of any cells. Although similar blanks have successfully yielded absorbance values close to control sample values for the FOX-1 and FOX-2 assays, this was not the case for the FOX-3 and L-FOX assays (Figure 3.5a). In particular, the absorbance for the FOX-3 blank was higher than both control and AAPH-treated samples. A further contrast with the FOX-1 and FOX-2 assays was provided by the observation that baseline absorbance readings varied dramatically between 0.15 to 0.4 absorbance units

from one experiment to the next (data not shown). This instability did not affect the degree of AAPH-induced hydroperoxide formation, so reproducible data could still be obtained simply by subtracting control absorbances from AAPH-treated absorbances (Figure 3.5b). These data manipulations enabled protein and lipid hydroperoxides to be calculated at concentrations of 0.66 ± 0.36μM and 2.46 ± 0.54μM, respectively, with lipid hydroperoxides accounting for 79% of all hydroperoxides. In comparison, the general PCA washing protocol yielded a hydroperoxide concentration of 1.66 ± 0.23μM (Figure 3.5b). This value differed from the protein hydroperoxide and lipid hydroperoxide results but was comparable to levels detected by the FOX-1 assay prior to lipid extraction (Figure 3.3). Some lipid was likely lost during the acid washing steps associated with both the general PCA protocol and the FOX-1 assay. Thus, the total (protein plus lipid) hydroperoxide content was not accurately measured in either of these two variations of the FOX assay and neither one was suitable for measuring hydroperoxide formation on HMDMs.

3.2.3 Time Course of AAPH-Induced Damage to HMDMs

Having identified appropriate assays for monitoring lipid and protein hydroperoxide formation, a time course of AAPH-mediated damage was then conducted. HMDMs were incubated with 20mM AAPH and, after an initial four hour lag, loss of cell viability was observed by both the MTT (Figure 3.6a) and trypan blue (Figure 3.6b) assays. Contrasting with the almost complete survival of control cells (particularly when monitored by trypan blue exclusion staining), virtually 100% of AAPH-treated cells were dead after 24 hours of exposure.

A lag phase of approximately four hours was also observed during AAPH-induced lipid oxidation (Figure 3.4 & Figure 3.6c). Similar trends were observed for both TBARS and lipid hydroperoxide formation, with a rapid increase in lipid peroxidation after the lag and a subsequent plateau in oxidation levels after 18 hours. Lipid hydroperoxide concentrations were consistently larger and, following an 18 hour incubation with 20mM AAPH, were at least 20 times greater than TBARS levels.

Lipid hydroperoxide formation was relatively linear between four and 18 hours, but occurred at a rate of only 0.15μM/hour, which is significantly below the concomitant rate of 22.97μM/hour for the generation of peroxyl radicals during AAPH decomposition. Nevertheless, lipid hydroperoxides are clearly a significant event of

HMDM exposure to AAPH, with concentrations becoming statistically different from zero hour levels within 12 hours ($p<0.01$). By contrast, protein hydroperoxides did not increase significantly above the zero hour measurement, even though their trend of formation was similar to that observed for lipid hydroperoxides (Figure 3.6c).

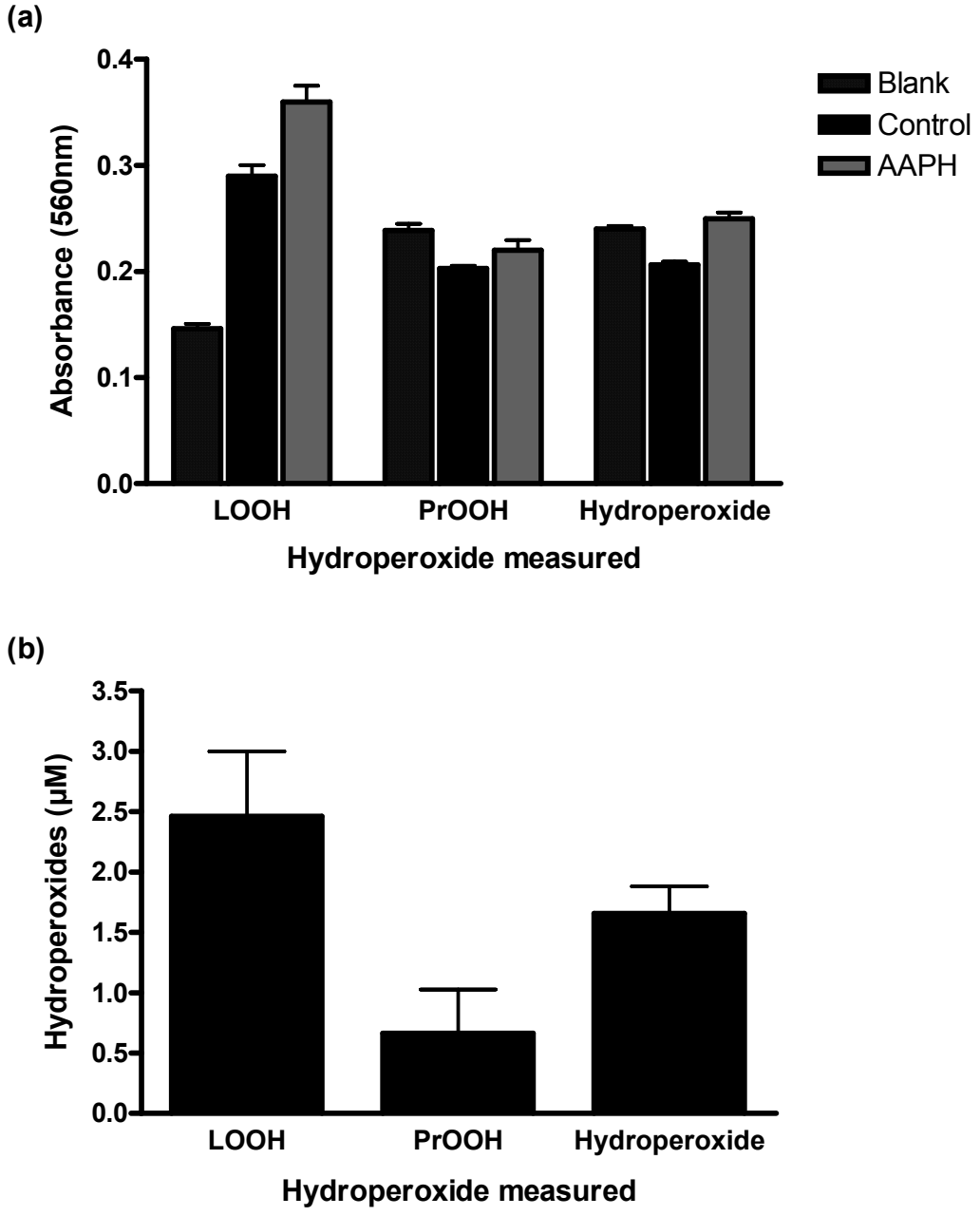


Figure 3.5 Characterization of hydroperoxides formed on HMDMs during AAPH-induced oxidation.

HMDMs ($5x10^6$ cells/ml) were incubated at 37°C in EBSS in the presence or absence (control) of 20mM AAPH. After 16 hours HMDMs were analyzed for lipid hydroperoxide (LOOH) and protein hydroperoxide (PrOOH) content using the L-FOX and FOX-3 assays, respectively. Blanks contained FOX reagents but no cells. A general measure of hydroperoxides was obtained using a modified version of the FOX-3 assay, with PCA washes but no chloroform washes. (a) Absorbances, measured at 560nm, were subsequently converted to (b) an AAPH-induced micromolar hydroperoxide concentration increase above the controls. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

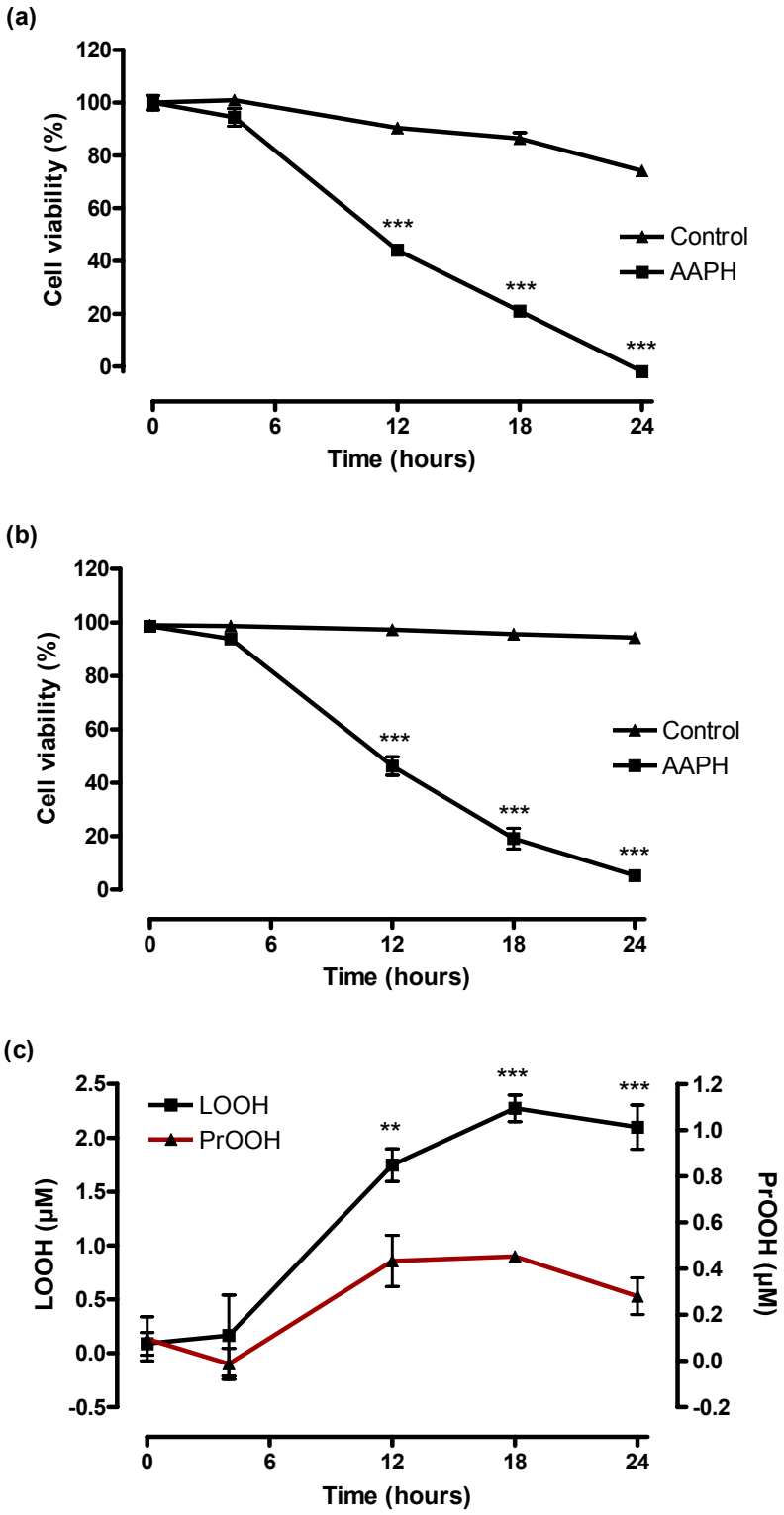


Figure 3.6 Time course of AAPH-induced damage to HMDMs.
HMDMs ($5x10^6$ cells/ml) were incubated at 37°C in EBSS in the presence or absence (control) of 20mM AAPH. At various times, HMDMs were analyzed for cell viability by both (a) MTT and (b) trypan blue exclusion staining, with data expressed as a percentage of zero hour levels. (c) The AAPH-induced increase in LOOHs and PrOOHs above the controls was also examined, using the L-FOX and FOX-3 assays, respectively. Significance is indicated from time zero. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments. In a separate experiment, cell viability was reduced to 81.0 ± 7.5% and 85.5 ± 1.1% via MTT and trypan blue exclusion staining, respectively, after six hours. This confirms that the lag phase ends after a four hour exposure to 20mM AAPH.

3.2.4 Effect of 7,8-NP on AAPH-induced damage to HMDMs

7,8-NP's potential antioxidant effect was studied by pre-incubating the HMDMs for ten minutes in EBSS with various concentrations of 7,8-NP. AAPH (20mM) was subsequently added and cells were incubated at 37°C for 12 hours before analyzing 7,8-NP's effect on viability and hydroperoxide formation (Figure 3.7). In agreement with Figure 3.1, 20mM AAPH reduced viability to approximately 50% after 12 hours. However, as determined by both MTT and trypan blue exclusion staining, the addition of up to 200μM 7,8-NP did not significantly protect against this AAPH-mediated cell death (Figure 3.7a & Figure 3.7b). 7,8-NP was also unable to prevent lipid hydroperoxide formation, although it did reduce protein hydroperoxide levels (Figure 3.7c). Despite this trend, the protection was not significant because protein hydroperoxides were not raised significantly above the control even in the absence of any 7,8-NP.

It is possible that the high flux of peroxyl radicals generated by 20mM AAPH overwhelmed any ability of 7,8-NP to inhibit cell death. This theory was investigated by incubating the HMDMs with a lower AAPH concentration of 10mM (Figure 3.8). Although the lower radical flux did ensure that 70-80% of HMDMs remained viable after a 12 hour incubation, 7,8-NP still failed to exert any protective effect against AAPH-mediated cell death.

Previous studies have found a correlation between 7,8-NP's ability to inhibit cell death and its prevention of cellular thiol loss (Duggan *et al.*, 2002; Baird *et al.*, 2004). Based on the above data (Figure 3.7 & Figure 3.8), it could therefore be theorized that 7,8-NP has no effect on the AAPH-induced oxidation of HMDM thiols. This potential association was investigated by using the DTNB assay to measure total cellular thiols following a 12 hour incubation of $5x10^6$ HMDMs/ml with 20mM AAPH and 7,8-NP (Figure 3.9). Control HMDMs contained approximately 25μM thiols and this level was reduced by 67% after exposure to 20mM AAPH. As observed for the cell viability studies, co-incubation with concentrations of up to 200μM 7,8-NP did not provide a significant protective effect against this thiol loss.

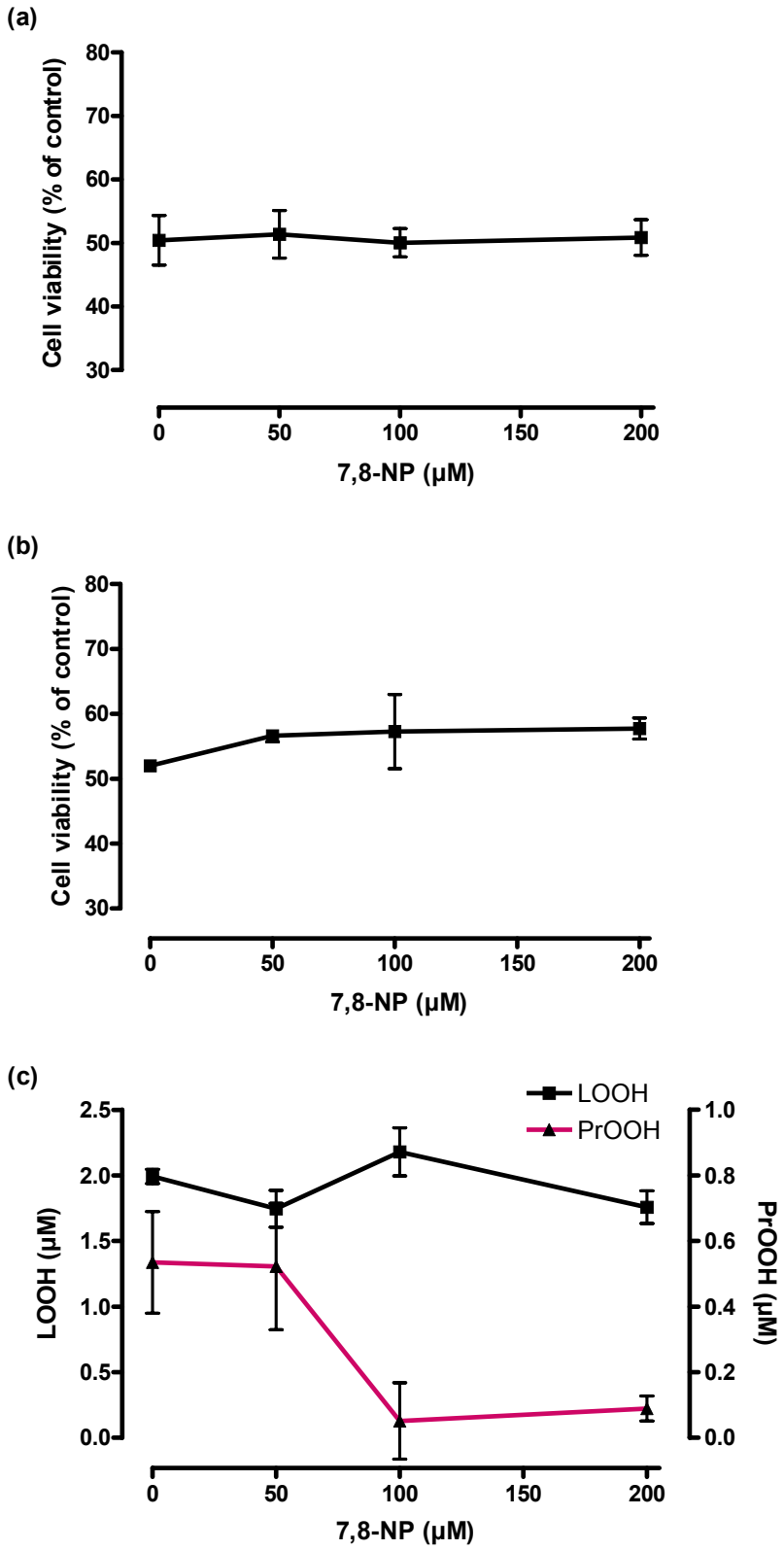


Figure 3.7 Effect of 7,8-NP on AAPH-induced oxidation of HMDMs.
HMDMs ($5x10^6$ cells/ml) were incubated at 37°C in EBSS with 20mM AAPH and increasing concentrations of 7,8-NP. Controls were conducted in the absence of AAPH. After 12 hours, HMDMs were analyzed for cell viability by both (a) MTT and (b) trypan blue exclusion staining. (c) LOOH and PrOOH levels were also monitored, using the L-FOX and FOX-3 assays, respectively, with controls subtracted from the AAPH-treated samples. ANOVA analysis revealed no statistical significance from 0µM 7,8-NP. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

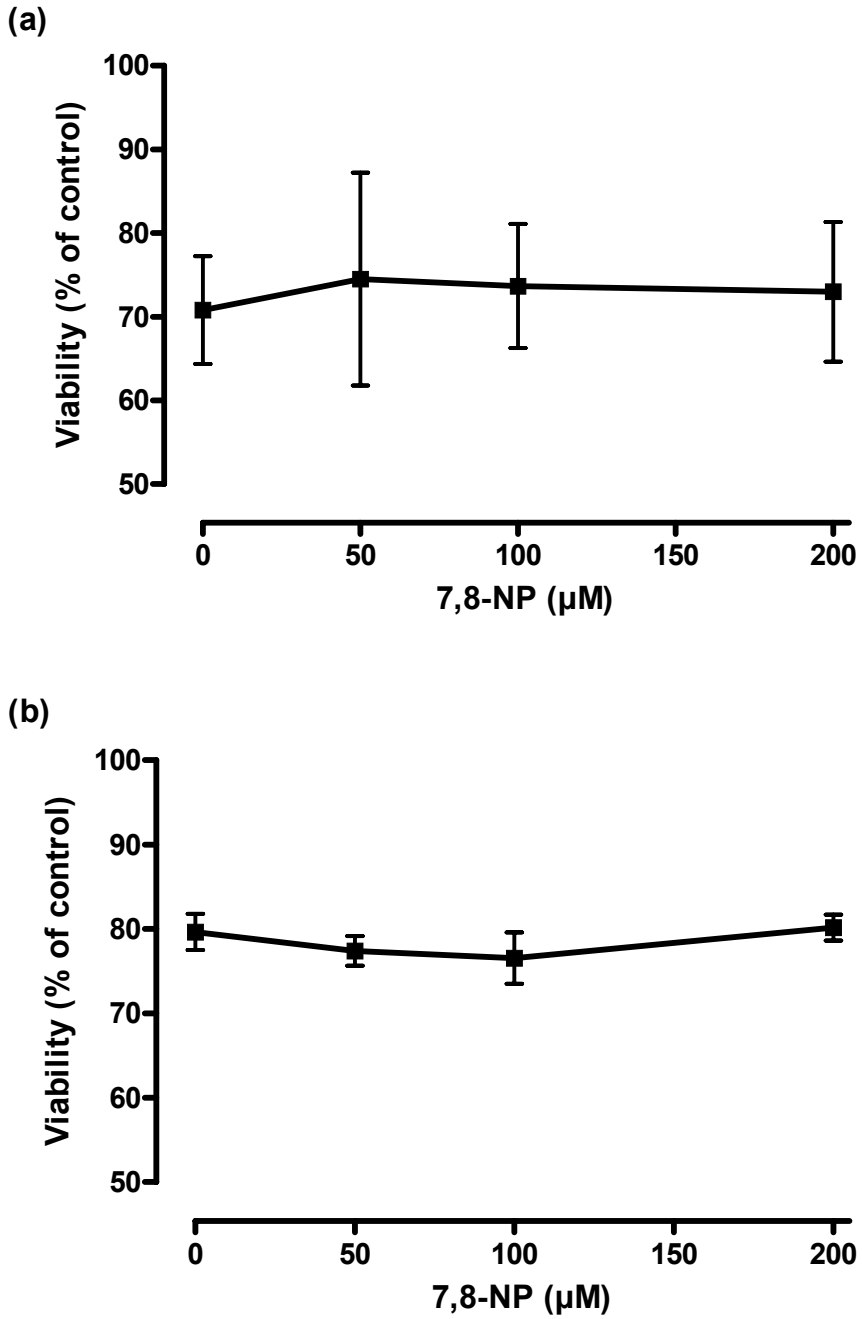


Figure 3.8 Effect of 7,8-NP on HMDM viability loss in the presence of 10mM AAPH.

HMDMs ($5x10^6$ cells/ml) were incubated at 37°C in EBSS with 10mM AAPH and increasing concentrations of 7,8-NP. Controls were conducted in the absence of AAPH. After 12 hours, HMDMs were analyzed for cell viability via (a) MTT and (b) trypan blue exclusion staining. ANOVA analysis revealed no statistical significance from 0µM 7,8-NP. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

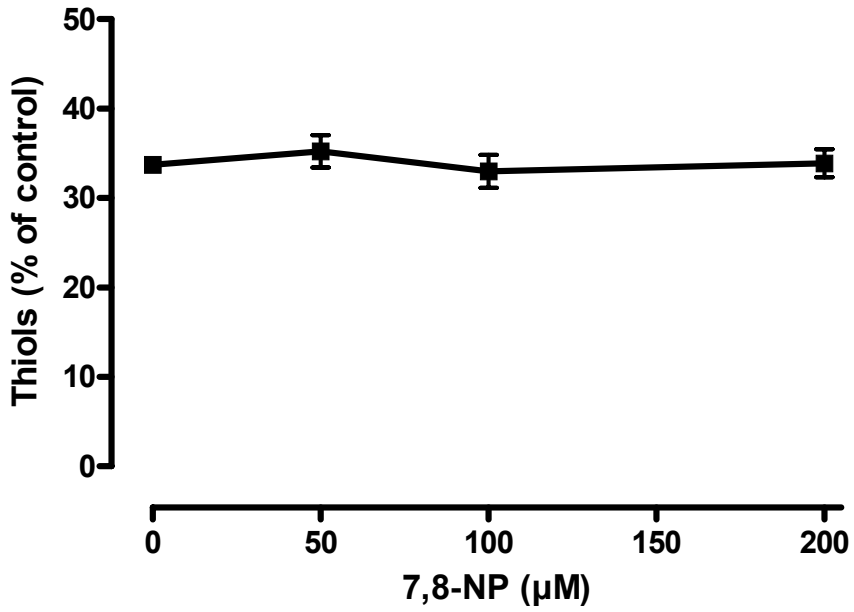


Figure 3.9 Effect of 7,8-NP on AAPH-induced thiol loss on HMDMs.
HMDMs ($5x10^6$ cells/ml) were incubated at 37°C in EBSS with 20mM AAPH and increasing concentrations of 7,8-NP. Controls were conducted in the absence of AAPH. After 12 hours, the total thiol content of HMDMs was analyzed by the DTNB assay. ANOVA analysis revealed no statistical significance from 0μM 7,8-NP. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

3.2.5 Effect of IFN-γ on AAPH-induced damage to HMDMs

While the results in section 3.2.4 clearly suggested that 7,8-NP offered no significant protection against AAPH-mediated damage to HMDMs, it remained possible that 7,8-NP would exert some effect when provided as an endogenous continuous supply rather than the exogenous bolus approach used above. IFN-γ is known to stimulate synthesis of 7,8-NP in macrophages (Huber *et al.*, 1984; Werner *et al.*, 1990), and a 48 hour pre-incubation was found to be optimal for 7,8-NP production (Figure 5.29). Thus, HMDMs were pre-incubated with various concentrations of IFN-γ for 48 hours before being washed and incubated in EBSS with fresh IFN-γ and 20mM AAPH. In agreement with the 7,8-NP experiments, no significant effect on cell viability or lipid hydroperoxide formation was observed (Figure 3.10). IFN-γ also did not inhibit generation of the minimal levels of protein hydroperoxides that were detected (Figure 3.10c).

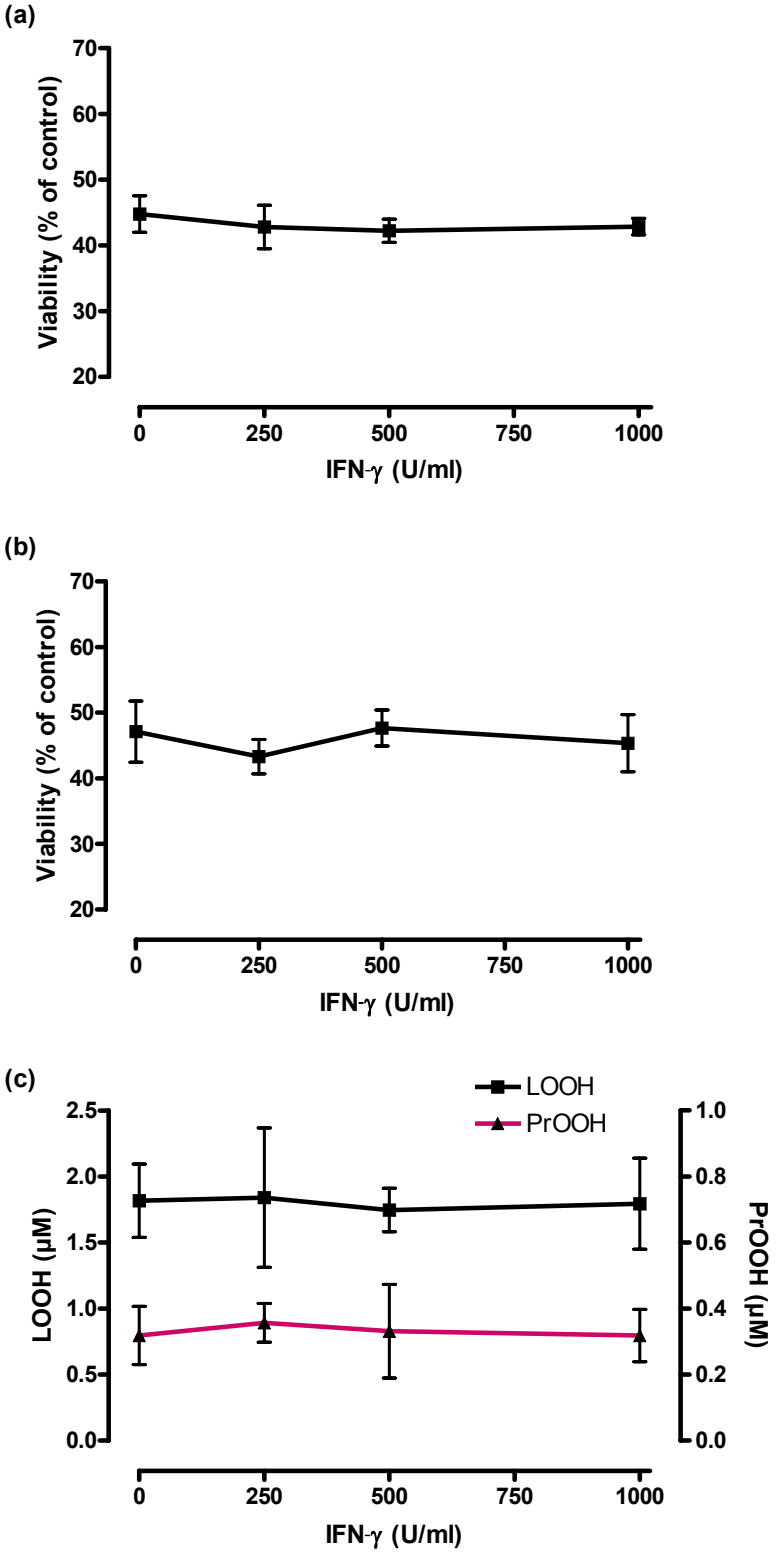


Figure 3.10 Effect of IFN-γ on AAPH-induced oxidation of HMDMs.
Following a 48 hour pre-incubation in RPMI at 37°C with increasing concentrations of IFN-γ, HMDMs ($5x10^6$ cells/ml) were washed and incubated for a further 12 hours in EBSS with 20mM AAPH and fresh IFN-γ. Controls were conducted in the absence of AAPH. HMDMs were analyzed for cell viability by both (a) MTT and (b) trypan blue exclusion staining. (c) LOOH and PrOOH levels were also monitored, using the L-FOX and FOX-3 assays, respectively, with controls subtracted from the AAPH-treated samples. ANOVA analysis revealed no statistical significance from 0U/ml IFN-γ. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

The lack of a concentration-dependent effect is not so surprising once the level of total pterin (neopterin and 7,8-NP) at each IFN-γ concentration is taken into account. Pterin, detected in both the supernatant and in the HMDM cellular extracts, did not vary significantly between 250 and 1000U/ml IFN-γ (Figure 3.11). Furthermore, the pterin levels dropped dramatically after replacing the RPMI medium with EBSS. This is partially due to the less nutrient rich nature of EBSS and the short incubation time of 12 hours, but exposure of the HMDMs to AAPH also accounts for the decrease. A 48 hour incubation with 500U/ml IFN-γ in RPMI, followed by 12 hours in EBSS without AAPH, yielded 36.3 ± 2.1nM and 62.6 ± 7.0pmole/mg protein of supernatant and cell extract pterin, respectively (data not shown). The supernatant concentration is twice as large as that obtained in the presence of 20mM AAPH, while the quantity of pterin measured in the cell extract is six times greater.

It should be noted that pterin concentrations showed some variability from one donor to the next. Most had pterin levels in the range displayed in Figure 5.29, with minimal or even no detectable pterins in the absence of IFN-γ and levels of up to 30nM of supernatant pterin and 35pmole/mg protein of cell extract pterin upon stimulation with IFN-γ for 48 hours. Concentrations shown in Figure 3.11 are significantly higher than this but were still unable to protect HMDMs from AAPH-induced cell death and hydroperoxide formation.

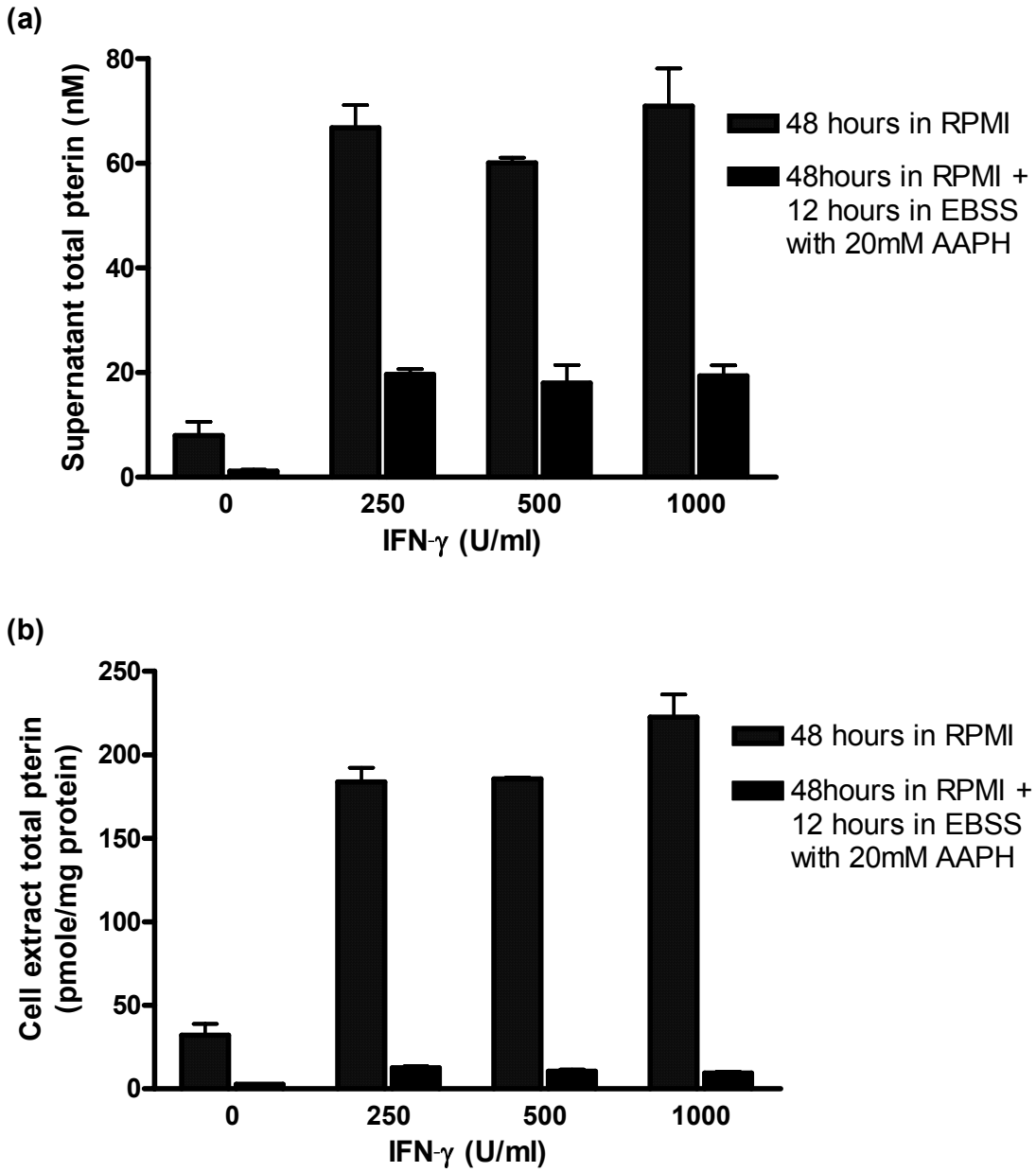


Figure 3.11 Pterin production by HMDMs exposed to increasing concentrations of IFN-γ.

HMDMs ($5x10^6$ cells/ml) were incubated at 37°C in RPMI with various concentrations of IFN-γ. After 48 hours, the supernatant and HMDM cell lysate in half the samples were analyzed for the presence of total pterin (neopterin plus 7,8-NP). HMDMs in the remaining samples were washed and incubated for a further 12 hours in EBSS with 20mM AAPH and fresh IFN-γ before measuring supernatant and cell lysate total pterin levels. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

3.3 Discussion

3.3.1 Three Day Culture Versus 14 Day Culture

AAPH induced a dose-dependent decrease in cell viability and this susceptibility was considerably more pronounced for the three day cultures (Figure 3.2) than the 14 day cultures (Figure 3.1). Similarly, tissue macrophages are more resilient towards ionizing radiation than circulating monocytes (Munn *et al.*, 1995). Differences between differentiated and undifferentiated cells have also been observed for the THP-1 cell line, with THP-1 macrophage-like cells consistently displaying an increased resistance towards oxidative stress compared to undifferentiated THP-1 monocytes when exposed to AAPH (Baird, 2003), oxLDL (Vicca *et al.*, 2000), and even hydrogen peroxide or a nitric oxide donor (Ferret *et al.*, 2000).

Differentiation of monocytes into macrophages involves not just a change in appearance but also a change in metabolism. Numerous studies have therefore been devoted to identifying genes that are either up-regulated or down-regulated during the differentiation process (for example, Asseffa *et al.*, 1993; Hart *et al.*, 1999; Kohro *et al.*, 2004; Whatling *et al.*, 2004). In particular, because macrophages have evolved to combat infection at sites of inflammation, it is not surprising that damaging reactive oxygen species are released in greater quantities from macrophages than monocytes (Mouithys-Mickalad *et al.*, 2001). A corresponding increase in endogenous antioxidants during differentiation is just as critical if macrophages are to survive under such oxidative conditions. Manganese SOD (Mn SOD) and copper-zinc SOD (CuZn SOD) are examples of antioxidant enzymes induced during differentiation (Ferret *et al.*, 2000; Pietarinen-Runtti *et al.*, 2000), while the vitamin E to total cholesterol ratio was also found to rise as HMDMs matured under *in vitro* conditions (Asmis & Jelk, 2000a). More extensive studies have revealed that glutathione levels are three-fold higher in THP-1 macrophages than monocytes, with depletion of this intracellular glutathione increasing the rate of oxLDL-induced cell death (Gotoh *et al.*, 1993). Differentiated THP-1 cells were also shown to produce 70% and 120% more thioredoxin and thioredoxin reductase mRNA, respectively, compared to undifferentiated cells (Ferret *et al.*, 2000). The same paper established a causal relationship between this antioxidant system and protection of the cells, by up-regulating thioredoxin expression via transfection, and subsequently observing increased resistance to oxidative stress.

In contrast to the above studies, some research has indicated that differentiation does not always lead to increased protection from oxidative stress. The PC12 rat neuronal cell line was more sensitive to oxidative stress once differentiated (Sasaki *et al.*, 2001), while U937 cells displayed no change either before or after differentiation (Ferret *et al.*, 2000). This apparent discrepancy was explained by a decrease in glutathione peroxidase activity and glutathione concentration upon differentiation in the PC12 cells and by a lack of any significant change in antioxidant activity in the U937s. These findings, therefore, ultimately provide further confirmation that resistance towards oxidative stress is correlated with cellular antioxidant activity. As such, the enhanced resistance of the 14 day cultures towards AAPH cytotoxicity when compared to the three day cultures is likely due to the increase in antioxidant activity that has previously been described upon differentiation.

Duggan (2000) reported that the exposure of mature macrophages to 10mM AAPH caused a 50-75% reduction in viability after 12 hours. This is comparable to the 10mM AAPH-induced drop in viability observed for three day cultures (Figure 3.2) but not for 14 day cultures (Figure 3.1), suggesting that the cells used in the Duggan study were not mature macrophages as initially stated. Indeed, later analysis of cells obtained using the methods described by Duggan (2000) showed that the majority were not fully differentiated (Gieseg & Duggan, personal communication).

Further support for the degree of AAPH-induced loss of viability observed in this study arises from the use of two distinct viability assays. The MTT assay measures the metabolic activity of cells (Mosmann, 1983), while the trypan blue assay monitors membrane integrity and is generally considered to be a less sensitive measure of a cell's state (Gieseg *et al.*, 2001b). Irrespective of their different mechanisms for detecting cell death, these two assays consistently yielded similar trend and percentage value data, and therefore provided strong corroboration of all viability results obtained.

3.3.2 Hydroperoxide Formation

Incubation with AAPH resulted not only in cell death but also in the generation of hydroperoxides on the HMDMs. Initial attempts to monitor hydroperoxide formation relied on the FOX-1 assay because this had been successfully used to monitor

AAPH-induced protein hydroperoxide formation in the U937 cell line (Gieseg *et al.*, 2000a). However, doubts were soon raised about the suitability of this assay for HMDM research, when it became apparent that the inclusion of a lipid extraction step dramatically lowered hydroperoxide levels (Figure 3.3). Similar results were obtained with all three solvent combinations tested (acetone/diethyl ether, methanol/hexane and methanol/chloroform), indicating that the decrease in absorbance was unlikely to be due to the use of a specific solvent combination. It was also unlikely to be due to trace amounts of solvent inhibiting the FOX assay because the incorporation of a 5% TCA wash after the solvent wash steps did not prevent the drop in absorbance.

The FOX-1 assay is suitable for detecting protein hydroperoxides only under conditions of minimal lipid hydroperoxide production and the above results suggested that this was not the case during exposure of HMDMs to AAPH. Although TBARS remained at low levels of approximately 0.1μM after 18 hours (Figure 3.4), this assay measures an end-product of lipid peroxidation rather than the lipid hydroperoxides themselves. In comparison, the L-FOX and FOX-3 assays are designed to directly detect lipid and protein hydroperoxides, respectively. The use of these assays revealed that approximately 79% of all hydroperoxides were located on the lipid rather than the protein component of HMDMs after a 16 hour incubation with 20mM AAPH (Figure 3.5). This contrasts with research exposing the U937 monocyte cell line (Gieseg *et al.*, 2000a) and red blood cell membranes (Firth, 2001) to AAPH, and it also contrasts with results obtained during gamma irradiation of both mouse myeloma Sp2/0 and U937 cells (Gebicki *et al.*, 2000b; Du & Gebicki, 2004). In all these studies, protein hydroperoxides rather than lipid hydroperoxides were detected.

Eukaryotic cells generally contain 74% protein versus 21% lipid and 4% DNA (Du & Gebicki, 2004). Thus, considering the abundance of protein in cells and also their susceptibility to free radicals, it is not surprising that cellular protein was the primary target of attack in these studies. Conversely, HMDMs are expected to be associated with large amounts of lipid because differentiation from blood-derived monocytes to macrophages has been reported to cause a 77-fold increase in total neutral lipid mass (Asmis & Jelk, 2000b). Such a dramatic accumulation of lipid during the culture of HMDMs produces cells with a foam-like appearance and has been attributed to the triglyceride-rich lipoprotein fraction of human serum (Garner *et al.*, 1997a). As the HMDMs in this study were cultured for a minimum of 14 days in the presence of 10% heat-inactivated human serum, they too were expected to become foam-like. This trait

was, indeed, microscopically observed. Of further relevance to this study, the peroxidizability of membrane lipids positively correlates with PUFA content (Alexander-North *et al.*, 1994). As a result, peroxidation is likely to be increased in HMDMs because the elevated lipid content is predominantly ascribed to long chain PUFAs of the n-3 series (Visioli *et al.*, 2000b). Thus, reasons similar to those used to explain the preferential attack of protein in U937s and mouse myeloma cells, can also be used to account for the current results. More specifically, this means hydroperoxides were predominantly detected on the lipid rather than the protein component of HMDMs due to the susceptibility of PUFAs to oxidative attack and their abundance in differentiated primary macrophages.

Primary cells are generally considered to be more physiologically relevant than cell lines but, because of their contrasting states of differentiation, direct comparisons between the U937 monocytes studied by Gieseg *et al*. (2000a) and the HMDMs are not entirely appropriate. Nevertheless, since blood-derived monocytes possess significantly lower levels of lipids than HMDMS, it can be speculated that protein may be the preferential target of attack prior to the differentiation of these primary cells.

This research also offers a note of warning against extrapolating directly to the *in vivo* situation. While primary cells may be more physiologically relevant than cell lines, the lipid profile of the HMDMs is nevertheless highly dependent on the conditions of culture. HMDMs cultured in media supplemented with human serum amass large quantities of lipid but HMDMs cultured in the presence of foetal calf serum do not (Garner *et al.*, 1997a). Whether AAPH-induced lipid hydroperoxide production would be reduced in HMDMs exposed to foetal calf serum is not currently known but could be investigated in future studies.

Unlike human serum, foetal calf serum is relatively deficient in triglycerides. By extension, it can be postulated that HMDMs cultured in human serum will accumulate lipid to a greater or lesser extent depending on the exact composition of the donor serum. The general *in vivo* significance of this serum-induced lipid accumulation in macrophages remains uncertain. It may, however, serve as a reasonable model for more specific situations, particularly those involving macrophage foam cells in the atherosclerotic plaque. Lipid profiles do differ slightly between foam cells isolated from plaque and HMDMs cultured in human serum (Garner *et al.*, 1997a). As plaque is considered to be a site of chronic inflammation and high oxidative stress (Van Lente, 2000; Scott, 2004), the detection of lipid hydroperoxides on HMDMs exposed to an

oxidant like AAPH raises the possibility of a similar scenario occurring in the atherosclerotic plaque. Lipid hydroperoxide formation on HMDMs in such an environment may be of significance to the atherogenic process for several reasons. For instance, macrophage foam cells are suggested to contribute to the necrotic core via the release of their lipids into the extracellular environment upon death (Ball *et al.*, 1995; Martinet *et al.*, 2001). Macrophages bearing peroxidized lipids on their membranes may also possess an additional means to promote the oxidation of LDL, with one study indicating that such macrophages promote LDL oxidation even in the absence of transition metals (Fuhrman *et al.*, 1994).

The specific location of the HMDM-associated lipid hydroperoxides was not investigated. However, it should be noted that AAPH is hydrophilic and has previously been shown to induce protein hydroperoxide formation on the cellular membrane rather than the intracellular components of U937 cells (Cassidy, 2003). While a similar hydroperoxide distribution may occur in HMDMs, the prospect of a lipid peroxidation chain reaction means that some damage may ultimately be transferred to the intracellular contents. Future studies could investigate this possibility by analyzing the various proportions of lipid hydroperoxides formed either in the HMDM cell extract or on the membrane following incubation with AAPH.

Kinetic analysis revealed that 20mM AAPH had no detectable effect on lipid hydroperoxide formation during the first four hours of incubation (Figure 3.6). Oxidation subsequently proceeded at a rate of 0.15μM lipid hydroperoxides per hour and then plateaued at 2.27 ± 0.12μM after 18 hours. By contrast, the equation described in section 3.1 indicates that 20mM AAPH incubated at 37°C generated peroxyl radicals at a steady rate of 22.97μM/hour. Thus, only one lipid hydroperoxide was formed per 152 peroxyl radicals. This is comparable to the ratio of one protein hydroperoxide per 200 peroxyl radicals calculated by Gieseg *et al.* (2000a) for U937 cells.

Any hydroperoxides detected do represent a balance between formation and degradation, so it should be noted that the lipid hydroperoxide to peroxyl radical ratio may be a slight underestimate. Furthermore, a low hit rate is not entirely unexpected because peroxyl radicals are likely to react not only with the HMDMs but with other peroxyl radicals and with any molecules in the medium as well. Other cellular

components also represent alternative targets, so even a reaction between peroxyl radicals and the cell will not necessarily generate lipid hydroperoxides.

Protein peroxidation is not a major outcome of peroxyl radical attack under the conditions of this study (Figure 3.6). The possibility remains that protein hydroperoxides were formed and then rapidly degraded but, considering that samples were collected throughout the incubation period, this seems unlikely.

By contrast, thiols appear to be important scavengers of peroxyl radicals. Only one third of total cellular HMDM thiols remained after a 12 hour incubation with 20mM AAPH (Figure 3.9). Antioxidants like thiols are certainly valid alternative cellular targets, with many exhibiting strong peroxyl radical scavenging activity. A reaction between peroxyl radicals and antioxidants would limit the extent of lipid hydroperoxide formation and could also explain the four hour lag phase observed prior to the acceleration of lipid peroxidation on HMDMs. A lag period observed during the AAPH-induced membrane oxidation of erythrocyte ghosts was attributed to α-tocopherol (Yamamoto *et al.*, 1985). The presence of ascorbate in sealed erythrocyte ghosts was also shown to provide antioxidant protection by regenerating the membrane-bound α-tocopherol (May *et al.*, 1998). Though conducted on a different cell type, these studies are of potential relevance because the α-tocopherol to total cholesterol ratio is known to increase in HMDMs during differentiation (Asmis & Jelk, 2000a). A lag in protein hydroperoxide formation has also been observed during AAPH-induced oxidation of the THP-1 monocyte cell line (Kappler, 2005). This protection was suggested to be mediated by glutathione because protein peroxidation and cell death were only initiated once the glutathione had been largely depleted. Considering the increase in glutathione that occurs during differentiation (Gotoh *et al.*, 1993), a similar scenario could account for the lag during AAPH-mediated damage to HMDMs. Likewise, thioredoxin levels also increase during macrophage differentiation (Ferret *et al.*, 2000), and this antioxidant system has been shown to inhibit apoptosis in glutathione-depleted lymphoid cells through maintenance of the cellular redox status (Iwata *et al.*, 1997).

Future studies could investigate the existence of such correlations in the HMDMs during AAPH-induced oxidative stress by analyzing the kinetics of antioxidant loss. Subsequent modulation of these antioxidant systems would then provide more direct evidence for their role in the lag phase.

A comparison of Figure 3.4 and Figure 3.6c confirmed that TBARS and lipid hydroperoxide formation yielded similar trends. In each case, a lag phase of approximately four hours was followed by a propagation phase and finally a plateau by 18 hours. This similarity is not surprising considering that both provide an indication of lipid oxidation, with lipid hydroperoxides directly monitoring the peroxidation process and TBARS measuring MDA, one of the aldehyde break-down products of lipid peroxidation. As such, it is also not surprising that TBARS levels are significantly lower than the lipid hydroperoxides themselves. In particular, by the time a plateau was reached at 18 hours, 2.27 ± 0.12μM lipid hydroperoxides but only 107 ± 3nM TBARS were detectable. Although it must be acknowledged that the lipid hydroperoxide concentration represents a balance between formation and break-down, it is still possible to obtain an approximate estimate of 5% of lipid hydroperoxides being decomposed to MDA.

In addition to elucidating the kinetics of AAPH-induced lipid oxidation on HMDMs, time course studies also highlighted a correlation between lipid and protein hydroperoxides, with protein hydroperoxides displaying a trend similar to that observed for lipid peroxidation (Figure 3.6c). Despite this, it should be noted that the protein hydroperoxide concentration on HMDMs never rose significantly above control levels.

Of more importance, therefore, may be the correlation observed between lipid hydroperoxide formation and loss of cell viability (Figure 3.6). In accord with the four hour lag observed during lipid peroxidation, both the MTT and trypan blues assays revealed an identical lag phase prior to any detectable cell death. This observation, combined with previous research, raises the possibility that these AAPH-induced lipid hydroperoxides contribute to the loss of viability in HMDMs. For instance, lipid hydroperoxides on oxLDL were shown to induce death in a variety of cell types, including human fibroblasts (Chisolm *et al.*, 1994; Coffey *et al.*, 1995) and human vascular smooth muscle cells (Siow *et al.*, 1999). Meanwhile, tert-butyl hydroperoxide (Masaki *et al.*, 1989) and 13-L-hydroperoxylinoleic acid (Aoshima *et al.*, 1987) triggered loss of viability in cultured hepatocytes and *Xenopus* oocytes, respectively, by a mechanism dependent on the lipid peroxidation of cellular membranes. This mode of cell death was suggested to be due to lipid hydroperoxides disrupting the membrane lipid bilayer and thereby increasing membrane permeability. Other studies have attributed lipid hydroperoxide-induced apoptosis to disruption of the cellular redox

balance, either via increased ROS production (Aoshima *et al.*, 1997) or a decrease in glutathione and the glutathione-to-oxidized glutathione ratio (Wang *et al.*, 2000). Lipid hydroperoxides have also been suggested to increase cytosolic calcium levels, thereby participating in the activation of a calcium-dependent apoptotic pathway (Sandstrom *et al.*, 1995; Li *et al.*, 2001). Such findings could be related to membrane disruption or to lipid hydroperoxide involvement in various signal transduction pathways (Girotti, 1998). Lipid hydroperoxides can also participate in damaging chain reactions, explaining why some studies have found apoptosis to be more directly linked to iron-dependent lipid peroxidation than to either calcium or glutathione levels (Tzeng *et al.*, 1995; Chiou *et al.*, 2003). The toxicity of lipid peroxidation end-products provides another means of inducing cell death, with 4-hydroxynonenal (HNE) able to directly induce apoptosis in human lens epithelial cells (Choudhary *et al.*, 2002).

The above studies indicate that lipid peroxidation can induce cell death in numerous ways, with the exact mechanism depending on the experimental conditions and cell type under investigation. A direct causal relationship between HMDM lipid hydroperoxides and loss of viability remains to be established but, once it is, additional research will clearly be required to identify their exact role in AAPH-mediated HMDM death.

3.3.3 Effect of 7,8-NP

Despite 7,8-NP's known ability to scavenge peroxyl radicals (Gieseg *et al.*, 1995; Oettl *et al.*, 1997), the addition of up to 200μM 7,8-NP failed to protect HMDMs from the cytotoxicity induced by 20mM AAPH (Figure 3.7a & b). This contrasts with the ability of micromolar quantities of 7,8-NP to significantly inhibit cell death in erythrocytes (Gieseg *et al.*, 2001a) and in U937 monocytes (Gieseg *et al.*, 2001b; Duggan *et al.*, 2002) during exposure of the cells to a range of oxidative insults.

Duggan *et al.* (2002) observed protection by seeding the U937s at a concentration of $5x10^6$ cells/ml (identical to the HMDM seeding concentration in this current study) but adding only 10mM rather than 20mM AAPH to the incubation medium. Although HMDMs display increased resistance towards AAPH compared to U937s, the use of different oxidant concentrations nonetheless raised the possibility of 7,8-NP's antioxidant activity simply being overwhelmed by the increased peroxyl radical flux. Incubating HMDMs for 12 hours with 10mM AAPH did reduce the radical flux by half,

leading to a corresponding decrease in HMDM death. However, 7,8-NP still failed to exert any protective effect under these conditions (Figure 3.8).

Although macrophages are known to release increased quantities of ROS compared to monocytes (Mouithys-Mickalad *et al.*, 2001), cellular antioxidants are correspondingly up-regulated too. As a consequence, 7,8-NP's lack of protective effect is unlikely to be due to any inability to offset the increased levels of oxidative stress associated with HMDMs. Of more significance, therefore, may be additional changes in metabolic response that are caused either by differentiation or by slight contrasts between the cell types. Several studies have discussed the influence of 7,8-NP on gene expression and cell signalling and it is possible that this compound's effect will vary depending on the metabolic state of the cell (for example, Woll *et al.*, 1993).

The structural changes that occur during differentiation in 10% human serum provide a more likely explanation for 7,8-NP's lack of effect during AAPH-mediated oxidation of HMDMs. Unlike U937s, HMDM cells are differentiated and lipid-loaded. Such differences may cause 7,8-NP and/or AAPH to interact or partition with the two cell types in distinct ways. One or the other may bind directly to, or be absorbed by, the HMDM cells in such a way that the 7,8-NP is unable to effectively scavenge radicals. In the absence of such interactions, the water soluble 7,8-NP would be expected to effectively scavenge the water soluble peroxyl radicals. This certainly appeared to occur when exposing BSA (Duggan *et al.*, 2001) or U937 cells (Duggan *et al.*, 2002) to AAPH and 7,8-NP, but was not observed during the current HMDM study. In further support of the interaction/partitioning theory, 7,8-NP provided some protection against AAPH-induced cytotoxicity in the presence of the less lipid-loaded and less differentiated primary macrophages studied by Duggan (2000).

In contrast with the protection mediated by 7,8-NP towards U937s, 7,8-NP had no effect on cellular thiols and only a limited impact on loss of viability in THP-1 cells (Baird *et al.*, 2005). THP-1 and U937 monocytes do not possess significant structural differences, so it is uncertain whether the interaction/partitioning theory is sufficient to account for the diverging results observed with these two cell lines. Regardless of this, the concept of an interaction between 7,8-NP and its substrates remains worthy of consideration. It has, in fact, already been discussed during LDL oxidation studies, with Gieseg *et al.* (1995) suggesting that 7,8-NP interacts with LDL in or on its lipid environment. This interaction enabled 7,8-NP to efficiently scavenge the LDL lipid peroxyl radicals that form and explains why 7,8-NP afforded similar levels of protection

against AAPH and copper attack, despite these compounds generating different types of radicals.

Whatever the precise mechanism(s) accounting for 7,8-NP's contrasting protectiveness towards a range of monocyte/macrophage cells, it is clear from AAPH and oxLDL studies on U937s that a positive correlation exists between 7,8-NP's ability to inhibit cellular thiol loss and its ability to inhibit cell death (Duggan *et al.*, 2002; Baird *et al.*, 2005). This correlation can be explained by considering the importance of glutathione, and thiols in general, for maintaining a healthy redox state within the cells. Given 7,8-NP's failure to prevent HMDM death, it was not surprising to discover that 7,8-NP also failed to inhibit AAPH-mediated oxidation of the total HMDM cellular thiols (Figure 3.9). Likewise, because lipid hydroperoxides are postulated to play a critical role in the AAPH-mediated death of HMDMs (section 3.3.2), it was anticipated that 7,8-NP would not prevent the generation of lipid hydroperoxides. A co-incubation with up to 200μM 7,8-NP caused no change in lipid hydroperoxide levels (Figure 3.7c), verifying this hypothesis and lending further support to the contribution of lipid hydroperoxides in HMDM death.

By contrast, 100μM 7,8-NP was sufficient to almost completely inhibit protein hydroperoxide formation after a 12 hour incubation with 20mM AAPH (Figure 3.7c). Due to the non-significant levels of protein hydroperoxides formed even in the absence of any 7,8-NP, this protection against AAPH is not statistically significant and it becomes difficult to make any strong conclusions. Nevertheless, the inhibition of protein hydroperoxides does agree with a previous study where 7,8-NP prevented AAPH-induced protein hydroperoxide formation on the U937 monocyte cell line (Duggan *et al.*, 2002). Furthermore, if 7,8-NP was scavenging AAPH-derived peroxyl radicals in the aqueous solution only, a similar level of inhibition would be expected for both lipid and protein hydroperoxides. Thus, the differential effect of 7,8-NP towards AAPH-mediated protein and lipid hydroperoxide formation supports the notion that 7,8-NP may interact with the HMDMs. This interaction may occur intracellularly and/or on the cell's surface but, irrespective of the location, it ultimately appears to place the 7,8-NP in a better position to scavenge the cells' protein peroxyl radicals and not the lipid peroxyl radicals.

The above results demonstrated an inhibition of protein hydroperoxide formation but no effect on thiol oxidation, lipid hydroperoxide formation or cell viability when 7,8-NP

was added in a single exogenous dose. Stimulation of HMDMs with IFN-γ was designed to ensure an endogenous and continuous supply of 7,8-NP. This, too, failed to prevent AAPH-mediated cell death and the generation of lipid hydroperoxides (Figure 3.10). Under these conditions there was also no inhibition of protein hydroperoxide formation, indicating that insufficient quantities of 7,8-NP were produced to provide the required protection.

Pterin (neopterin and 7,8-NP) levels were certainly lower following the 12 hour incubation with 20mM AAPH in EBSS compared to the preceding 48 hour incubation in RPMI (Figure 3.11). Several reasons account for this dramatic drop, including the fact that EBSS is less nutrient rich than RPMI and would have contributed to a slower rate of 7,8-NP synthesis. Furthermore, all 7,8-NP released into the RPMI was lost once the incubation medium was changed to EBSS. As cells were subsequently incubated in EBSS for only 12 hours, there was less time for the 7,8-NP levels to build up again. Although pterin levels may have been higher if the entire pre-incubation and incubation were conducted in EBSS, this is not feasible when using EBSS. Sixty hours in minimal medium is likely to adversely affect the health of cells even in the absence of AAPH.

Ultimately, AAPH may play the largest role in accounting for the dramatic decrease in pterins. While transferral to EBSS did reduce supernatant and intracellular pterin levels, the addition of 20mM AAPH decreased the yields even further. Thus, the stress and cell death caused by AAPH was clearly more than sufficient to adversely affect 7,8-NP synthesis. Whether HMDMs would be better able to synthesize 7,8-NP, and provide protection, in the presence of a lower level of oxidative stress is unclear. However, with respect to AAPH, this seems unlikely. Halving the dose of AAPH to 10mM did reduce the extent of cell death to approximately 25% but the bolus addition of up to 200μM 7,8-NP still failed to protect against this lower level of oxidative damage (Figure 3.8). Moreover, the lack of protection observed in this study with IFN-γ may not be restricted to an oxidant like AAPH. Baird (2003) showed that IFN-γ was also unable to prevent oxLDL-induced death in a range of cell lines, including U937 monocytes, THP-1 monocytes and THP-1 macrophage-like cells. This lack of protection was observed despite the entire experiment (including a 24 hour pre-incubation) being conducted in RPMI supplemented with 300U/ml IFN-γ.

The absence of any concentration-dependent effect of IFN-γ on AAPH-mediated damage is, in itself, not unexpected because pterin levels displayed no reliance on IFN-γ between the range of 250-1000U/ml (Figure 3.11). This range of concentrations was

chosen based on findings by previous studies of a positive correlation between IFN-γ and pterin levels (Werner-Felmayer *et al.*, 1990; Gruber *et al.*, 2000). The apparent disparity between these results and the current study may simply be due to cell type-specific differences, with the THP-1 cell line being used previously compared to HMDMs now. Pterins released from human macrophages have been reported to exhibit a concentration-dependence towards IFN-γ. However, this effect was only observed up to 100U/ml IFN-γ, with pterin levels reaching a plateau thereafter (Huber *et al.*, 1984). This agrees with the plateau detected between 250 and 1000U/ml IFN-γ in Figure 3.11, and provides another cautionary tale against extrapolating directly from cell line data to primary cells. The result does not detract from the fact that, under the conditions of this study, stimulation with IFN-γ failed to protect HMDMs from AAPH-mediated damage.

3.4 Summary

The differentiation of blood-derived monocytes produced HMDMs possessing an increased resistance towards AAPH-induced oxidative stress. Double the dose of AAPH was required to kill 50% of 14 day cultures compared to three day cultures under otherwise identical experimental conditions. Kinetic analysis revealed that the AAPH-mediated death of HMDMs was closely correlated to lipid hydroperoxide formation. Both parameters displayed a lag of approximately four hours before the initiation of any detectable damage and, given the findings of previous studies, it seems likely that lipid peroxidation contributes to the death of HMDMs. This theory is further supported by the inability of up to 200μM 7,8-NP to prevent both loss of viability and lipid hydroperoxide formation. Protein hydroperoxides are unlikely to play a major role in HMDM death because, although a similar kinetic trend was observed, protein hydroperoxide levels remained low and never reached significance. Furthermore, 100μM 7,8-NP was sufficient to inhibit protein hydroperoxide formation, while having no effect on cell viability.

Lipid rather than protein appears to be the primary target of peroxyl radical attack on HMDMs, presumably due to the fact that HMDMs accumulate PUFA-rich lipid and become foam-like in appearance. This structural change is also postulated to affect the way 7,8-NP interacts with the HMDMs, limiting its effectiveness as a radical scavenger and thereby preventing 7,8-NP from inhibiting AAPH-mediated thiol loss,

lipid hydroperoxide formation and, ultimately, cell death. This lack of effect by 7,8-NP towards HMDMs exposed to AAPH bears more resemblance to the THP-1 cell line than to the U937 cell line.

Stimulation with IFN-γ also failed to inhibit hydroperoxide formation and cell death in the presence of 20mM AAPH. Thus, under the conditions of this study, both a single exogenous addition and a continuous endogenous supply of 7,8-NP were incapable of protecting HMDMs from AAPH-mediated damage.

4 Ham's F10 Medium and LDL Oxidation

4.1 Introduction

Ham's F10 is a popular choice of medium for experiments studying cell-mediated LDL oxidation (for example, Sparrow & Olszewski, 1993; Maziere *et al.*, 1995; Wood & Graham, 1995; Halvorsen *et al.*, 1996; Marchant *et al.*, 1996; Carpenter *et al.*, 1997; Muller *et al.*, 1998; Gieseg & Cato, 2003) and has several advantages when compared to other types of media. It is more nutrient-rich than the minimal media, HBSS and EBSS, but less enriched than RPMI. This balance enables Ham's F10 to effectively maintain cells in a healthy state for several days but, conversely, limits the level of potential antioxidant compounds (amino acids, thiols and vitamins) that could inhibit the pro-oxidant activity of the incubating cells (Faure *et al.*, 2004). For this reason, Ham's F10 requires supplementation with a lower level of transition metal ions than RPMI 1640 to achieve an equivalent level of cell-mediated LDL oxidation (van Reyk *et al.*, 1999). It is also less likely than RPMI to interfere with the antioxidant activity of any compounds studied (Faure *et al.*, 2004).

These features make Ham's F10 ideal for the current research; an investigation of 7,8-NP's capacity to inhibit macrophage-mediated LDL oxidation. Unfortunately, the quality of commercially available Ham's F10 media appears to have diminished in recent years. Problems in LDL oxidation studies coincided with the depletion of a batch of Gibco BRL's Ham's F10 liquid medium. Gibco BRL had, by this point, halted the manufacture of Ham's F10 for a couple years so the media had to be replaced with what was assumed to be the powdered equivalent from Sigma. Of particular concern was the detection of sudden inconsistency in the rates of cell free LDL oxidation. By contrast, cell-mediated rates of LDL oxidation remained relatively constant. Although there were no obvious changes in the chemical composition of Ham's F10, informal discussions with other labs supported the idea of an intrinsic but unidentified inconsistency in the media (A. Baoutina; K. Carpenter, personal communications).

A cell free (LDL only) control is important because a basal level of oxidation occurs whenever transition metals are present. Cells are considered to enhance this process. Consequently, an ideal experimental set-up for monitoring cell-mediated LDL oxidation should result in minimal cell free oxidation while still maintaining a significant level of cell-mediated oxidation. Unpredictable changes in the rate of cell free LDL oxidation

make it difficult to systematically study cell-mediated LDL oxidation and any ensuing inhibition that occurs during 7,8-NP or IFN-γ treatment.

This section of work therefore highlights the problems experienced during cell free LDL oxidation and provides an overview of the steps taken to identify the cause of the inconsistency and alleviate the problem.

4.2 Results

4.2.1 Variability in the Rate of Cell Free LDL Oxidation

Inconsistencies between experiments became apparent when monitoring the rate of cell free LDL oxidation, with the lag phase varying from anywhere between three hours to over 24 hours (Figure 4.1 & Figure 4.2). This variability was observed whether measuring protein oxidation, lipid oxidation or even vitamin E loss. Meanwhile, cell-mediated LDL oxidation maintained relatively consistent rates between experiments. This led to large variations in the length of time that cell free samples remained significantly less oxidized than the cell plus LDL samples, with only one time point of significance in Figure 4.1 versus all time points between six and 24 hours in Figure 4.2. In some experiments, the cell free oxidation proceeded so fast that there was no significant difference between incubations in the presence and absence of cells (data not shown).

Initial observations indicated that the rate of oxidation was generally slower when using fresher batches of media. The addition of 25mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) also appeared to delay the sudden onset of variability observed between experiments. However, even these trends could not be guaranteed. Some of the fastest cell free LDL oxidations were observed when using HEPES-supplemented Ham's F10, prepared only a few days prior to the experiment (data not shown).

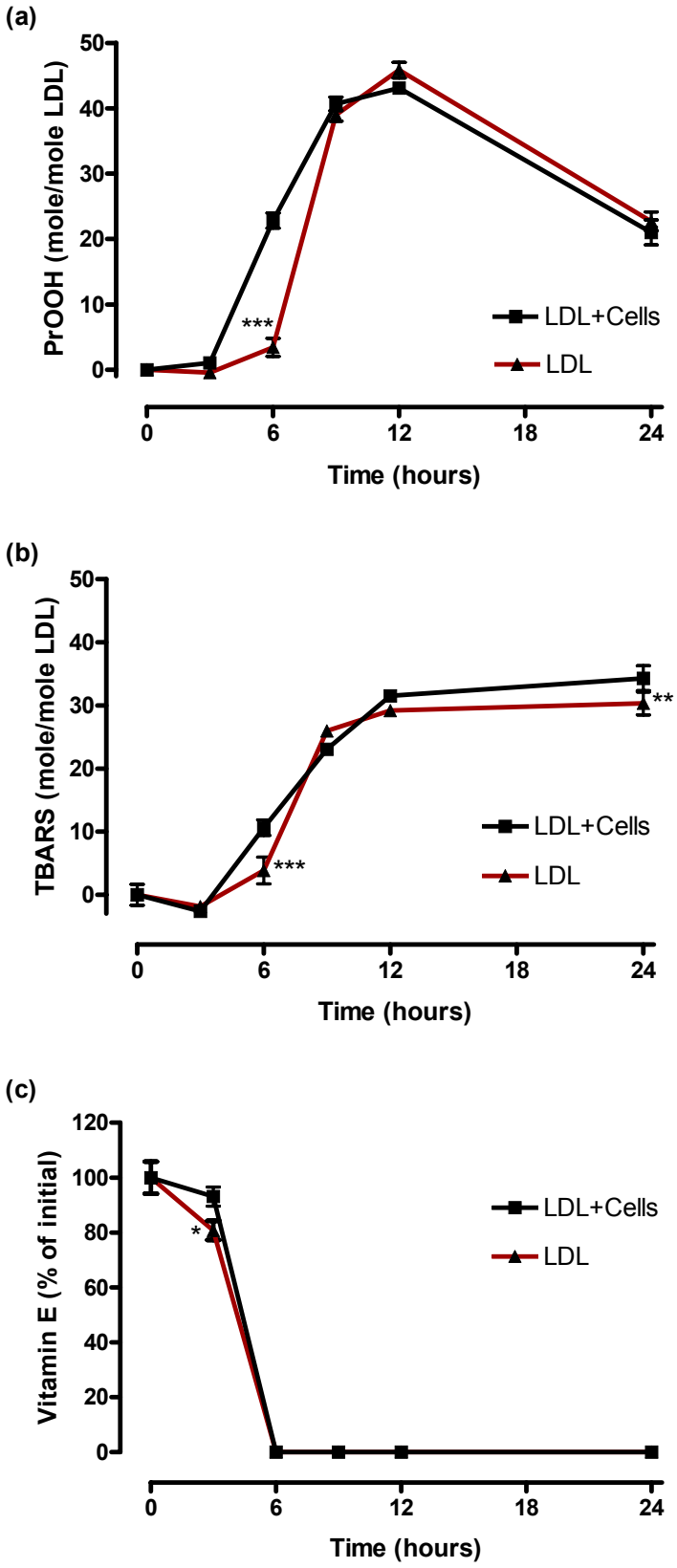


Figure 4.1 Rapid cell free LDL oxidation in Ham's F10 medium.
LDL (0.1mg/ml) was incubated at 37°C in Ham's F10, supplemented with 25mM HEPES and 1.5µM $FeSO_4$, in the presence or absence of adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml). Samples were analyzed at various time points for an increase in (a) protein hydroperoxides and (b) TBARS and (c) a loss of vitamin E. Significance is indicated between treatments. Results are displayed as mean ± SEM of triplicate samples.

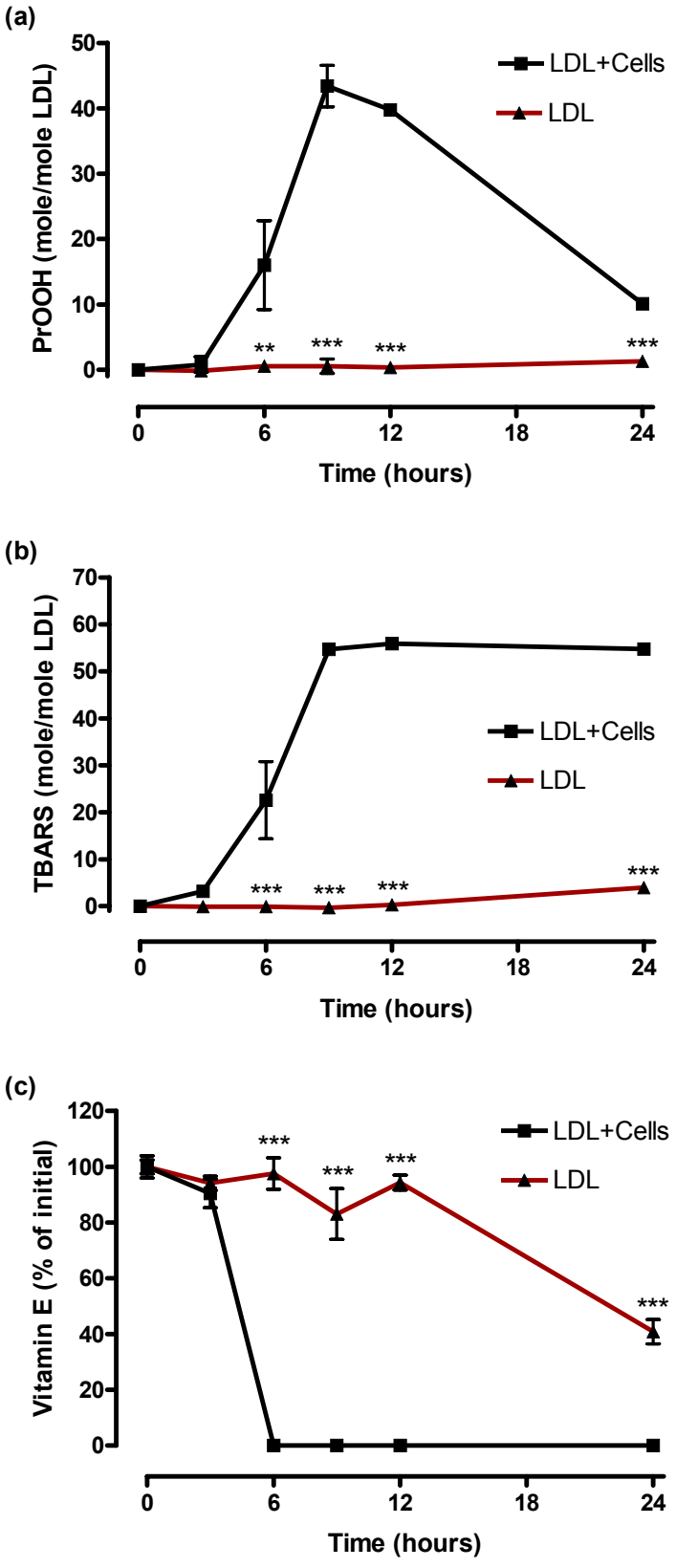


Figure 4.2 Slow cell free LDL oxidation in Ham's F10 medium.
LDL (0.1mg/ml) was incubated at 37°C in Ham's F10, supplemented with 25mM HEPES and 1.5µM $FeSO_4$, in the presence or absence of adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml). Samples were analyzed at various time points for an increase in (a) protein hydroperoxides and (b) TBARS and (c) a loss of vitamin E. Significance is indicated between treatments. Results are displayed as mean ± SEM of triplicate samples.

The role of media in the cell free oxidation inconsistency was more rigorously tested by setting up four LDL oxidation treatments that differed only in the Ham's F10 (Figure 4.3). Two month old Medium 1a lacked HEPES, while one month old Medium 2a was supplemented with 25mM HEPES at the time of media preparation. Despite the lack of any other differences, a large discrepancy in the degree of cell free LDL oxidation was observed between the two treatments. The addition of fresh HEPES to the two month old Medium 1a served as the basis for Medium 1b but this had only a limited impact on the level of cell free LDL oxidation at 12 and 24 hours. By contrast, Medium 2b permitted significantly more TBARS formation on LDL than Medium 2a after a 24 hour incubation. Both were prepared on the same day and from the same batch of powdered Ham's F10 but merely stored in different bottles. Medium 2b's exposure to oxygen and light was also limited by leaving it undisturbed in an unopened, darkened bottle for the entire month after preparation. (All other bottles were partially empty.) The low level of cell free LDL oxidation in Medium 2a differs not only from that obtained with Medium 2b but also with the trend observed using Medium 2a two weeks earlier. In that experiment, TBARS concentrations of 13.41 ± 1.79 and 42.22 ± 1.07 moles/mole LDL were detected after 12 and 24 hours, respectively (data not shown).

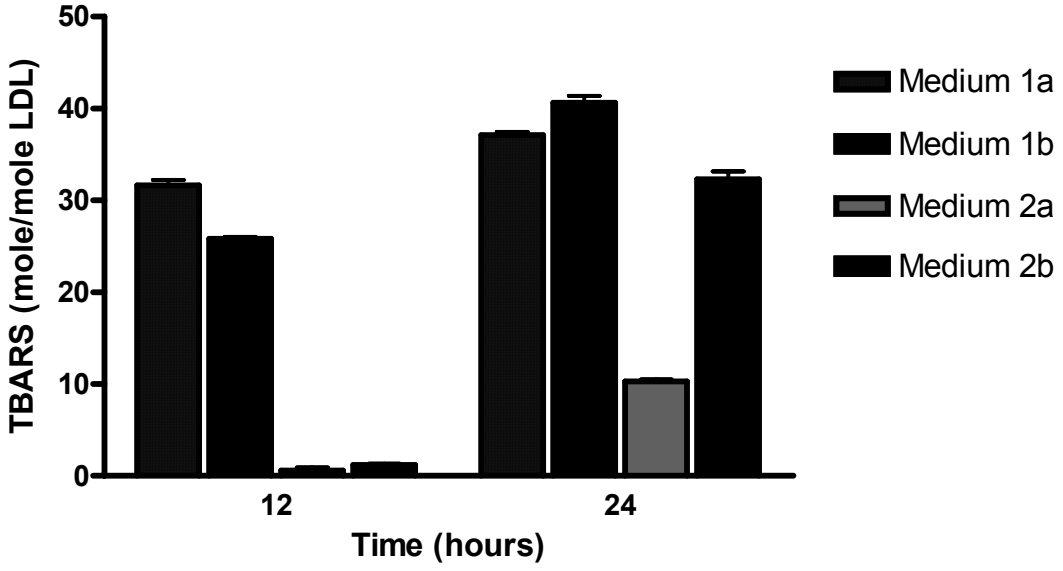


Figure 4.3 Effect of different Ham's F10 media on cell free LDL oxidation.
LDL (0.1mg/ml) was incubated at 37°C in Ham's F10 supplemented with 1.5µM $FeSO_4$. After 12 and 24 hours, samples were analyzed for an increase in TBARS. Medium 1a: Two month old Ham's F10, lacking HEPES. Medium 1b: Same batch as medium 1a but with 25mM HEPES added immediately prior to the experiment. Medium 2a: One month old Ham's F10, containing 25mM HEPES. Medium 2b: Same batch as medium 2a, containing 25mM HEPES, but stored in an unopened, darkened bottle for the entire month. Results are displayed as mean ± SEM of triplicate samples.

4.2.2 Medium Modifications

Variable rates of cell free LDL oxidation continued to be observed after preparation of the Ham's F10 in water sourced from an alternative filtration system (data not shown). Even purchasing the medium from an alternative supplier failed to solve the inconsistency problem, as was confirmed when Gibco BRL began reselling Ham's F10 (Table 4.1). A 12 hour incubation in Gibco BRL Ham's F10 yielded concentrations of LDL-associated protein hydroperoxides that differed significantly between April and July 2004 ($p<0.001$). The variability associated with this Gibco BRL medium contrasts with the consistent cell free results obtained when using Ham's F10 purchased from Gibco BRL a few years before the current problems. However, it agrees with the inconsistency observed during cell free LDL oxidation in Sigma Ham's F10. This similarity to Sigma Ham's F10 occurs despite the two sources of media having distinctly different preparation and storage procedures. Ham's F10 from Gibco BRL was purchased in liquid form and stored in sterile plastic containers supplied by the medium manufacturer. Sigma Ham's F10 was purchased as a powder and then prepared and stored in sterile glass bottles sourced from the Free Radical Biochemistry Laboratory.

Table 4.1 Cell free LDL oxidation in Ham's F10 medium sourced from Gibco BRL.
LDL (0.1mg/ml) was incubated at 37°C in Ham's F10, sourced from Gibco BRL, and supplemented with 1.5μM $FeSO_4$ and 25mM HEPES. After 12 hours, samples were analyzed for an increase in protein hydroperoxides. The experiment was conducted on two different occasions (April 2004 and July 2004) but using the same bottle of Ham's F10 medium. Significance is indicated between dates. Results are displayed as mean ± SEM of triplicate samples.

	April 2004	July 2004
Protein hydroperoxide (mole/mole LDL)	3.84 ± 0.64	33.54 ± 2.26 ***

Dr Keri Carpenter (personal communication) has also experienced a lack of consistency during LDL oxidation in Ham's F10 but was largely able to solve the instability by supplementing phenol red to a concentration of 12mg/L. Sigma and Gibco BRL Ham's F10 currently contain low quantities of 1.3mg/L and 1.2mg/L phenol red, respectively, but prior to 1994, contained the much higher concentration of 12mg/L

(Wilkins & Leake, 1994). However, increasing the phenol red concentration to pre-1994 levels failed to reproduce Dr Carpenter's result (Figure 4.4). No inhibition was observed compared to unsupplemented Sigma Ham's F10. The supplemented treatment actually appeared to promote a slightly faster rate of cell free LDL oxidation, though this was not significant for protein hydroperoxide formation and only significant for vitamin E loss at six hours ($p<0.05$).

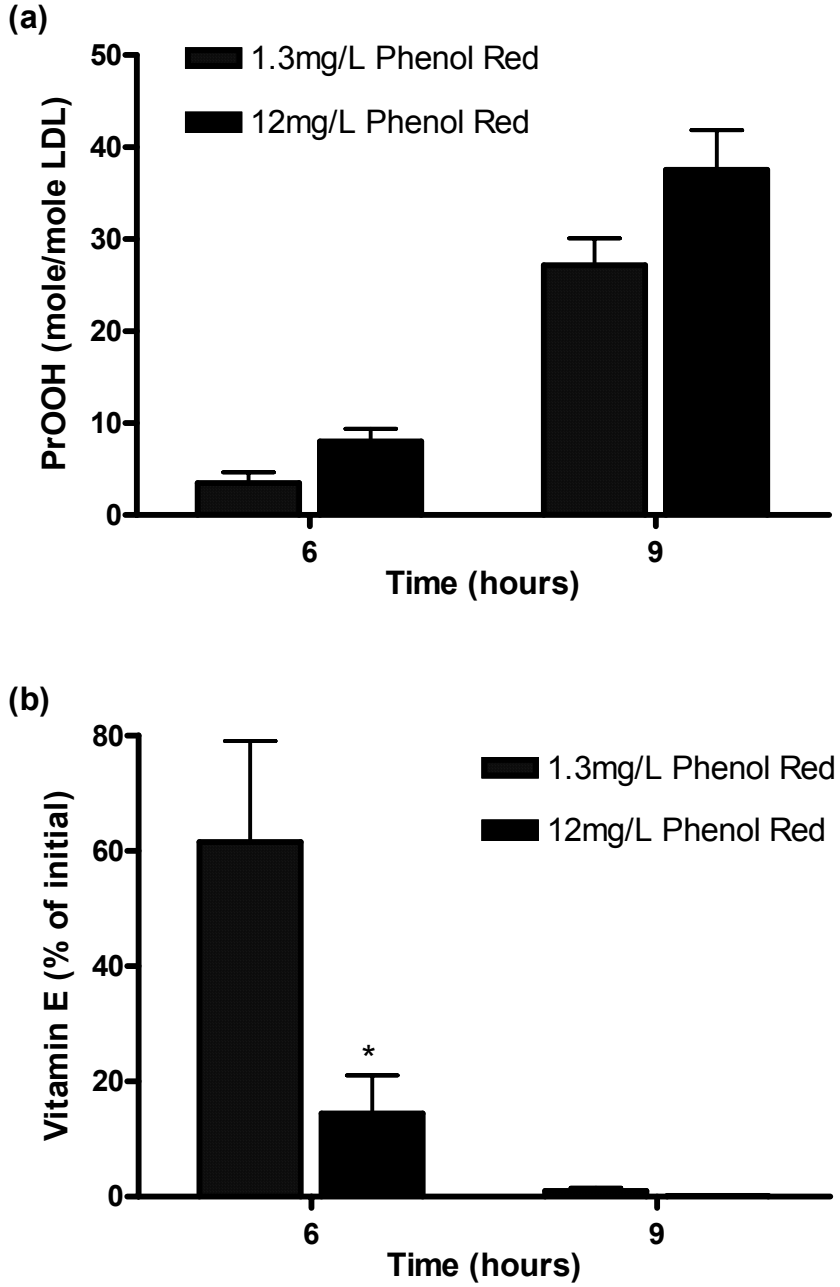


Figure 4.4 Effect of phenol red on cell free LDL oxidation.
LDL (0.1mg/ml) was incubated at 37°C in Ham's F10, containing 25mM HEPES and either 1.3mg/L or 12mg/L phenol red. All media were supplemented with 1.5µM $FeSO_4$. After both six and nine hours, samples were analyzed for (a) an increase in protein hydroperoxides and (b) a loss of vitamin E. Significance is indicated between treatments. Results are displayed as mean ± SEM of triplicate samples.

Having failed to eliminate the cell free inconsistency by antioxidant supplementation, an attempt was then made to identify and subsequently modify the component(s) in Ham's F10 responsible for the variability. Thiols in the media were initially studied because of their ability to contribute to LDL oxidation but, regardless of this potential, no clear correlation was found to exist between Ham's F10 thiol levels and the extent of cell free LDL oxidation (Figure 4.5). While Medium 1 did contain the lowest quantities of both thiols in the Ham's F10 and protein hydroperoxides on the incubated LDL, the other media contradicted this trend. Protein hydroperoxide formation was considerably more pronounced when LDL was incubated in Medium 3 compared to Medium 2 ($p<0.001$) even though these two media exhibited no significant difference in thiol concentrations.

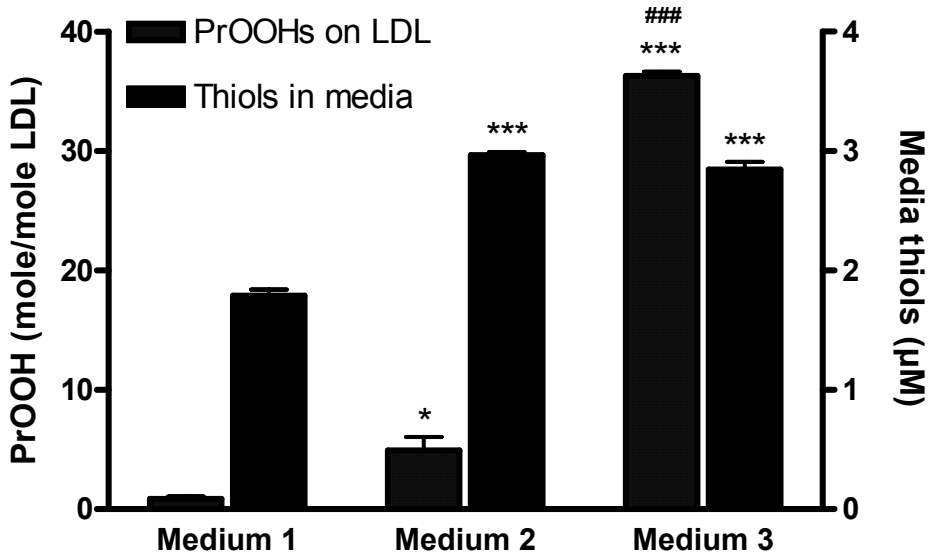


Figure 4.5 Comparison between thiol levels in Ham's F10 media and cell free protein hydroperoxide formation on LDL.

Thiol levels were measured in each of three batches of Ham's F10 media before supplementing with 1.5μM $FeSO_4$ and incubating with 0.1mg/ml LDL at 37°C. After nine hours, samples were analyzed for an increase in protein hydroperoxides. Medium 1: Five month old Ham's F10, containing 25mM HEPES. Medium 2: Four month old Ham's F10, lacking HEPES. Medium 3: One week old Ham's F10, lacking HEPES. * indicates significance from Medium 1. # indicates significance from Medium 2. Results are displayed as mean ± SEM of triplicate samples.

Like thiols, glutamine also deserved consideration as a source of the cell free instability. Admittedly, the literature does not refer to a role for glutamine in LDL oxidation but, at a concentration of 1mM, it is the most abundant amino acid present in Ham's F10. Just as critically, it has been shown to degrade over time (Sigma product information sheet). Ham's F10 was therefore supplemented with 1mM glutamine immediately before the initiation of a cell free LDL oxidation experiment (Figure 4.6). The supplemented treatment failed to show any significant difference in LDL-associated TBARS compared to the unsupplemented control after either six or nine hours of cell free oxidation.

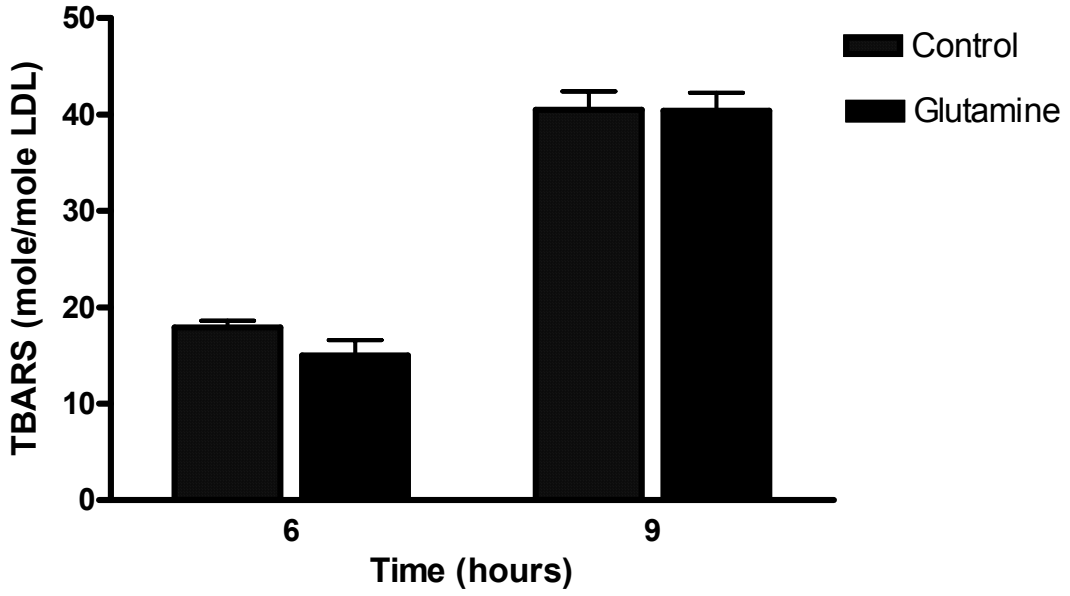


Figure 4.6 Effect of supplementation with fresh glutamine on cell free LDL oxidation.
LDL (0.1mg/ml) was incubated at 37°C in Ham's F10, containing 25mM HEPES. All media was supplemented with 1.5μM $FeSO_4$ but only half was supplemented with 1mM fresh glutamine. After both six and nine hours, samples were analyzed for an increase in TBARS. ANOVA analysis revealed no statistical significance between treatments. Results are displayed as mean ± SEM of triplicate samples.

Iron plays a critical role in LDL oxidation under the conditions of this study, raising the possibility that it may somehow contribute to the inconsistent rates of cell free LDL oxidation. Initial studies had indicated that a supplemental ferrous sulphate concentration of 1.5µM was required for optimal results (Figure 5.1 to Figure 5.3). However, ferrous sulphate is not the only suitable source of supplemental iron for LDL oxidation studies. Ferric chloride also has the potential to promote LDL oxidation and has, in fact, been used by another research group without any major problems (W. Jessup, personal communication). Thus, the stability of cell free LDL oxidation after supplementation with 1.5µM ferric chloride was investigated over a period of five days and compared to oxidation in the presence of 1.5µM ferrous sulphate (Figure 4.7). Despite such a short time frame, supplementation with ferric chloride showed dramatic variations in the extent of cell free LDL oxidation ($p<0.001$). The values differed so widely that, although a significant difference in the extent of TBARS formation was apparent between ferric chloride and ferrous sulphate treatments after the first experiment ($p<0.001$), this difference was no longer significant after the second experiment.

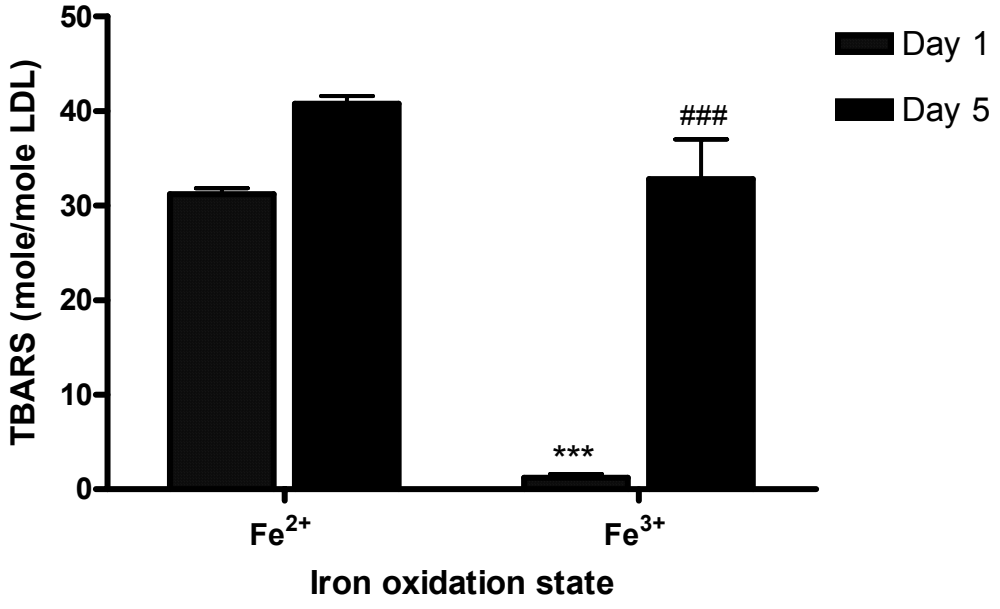


Figure 4.7 Effect of different iron oxidation states on cell free LDL oxidation.
LDL (0.1mg/ml) was incubated at 37°C in Ham's F10, containing 25mM HEPES, and supplemented with either 1.5µM $FeSO_4$ or 1.5µM $FeCl_3$. After 24 hours, samples were analyzed for an increase in TBARS. The experiment was conducted on two different occasions (day 1 and day 5) but using the same bottle of Ham's F10 medium. * indicates significance between the different iron treatments. # indicates significance between dates. Results are displayed as mean ± SEM of triplicate samples.

Supplementary iron is not the only source of metal in these LDL oxidation experiments because the Ham's F10 is formulated with 3µM ferrous sulphate. This iron(II) is likely to oxidize over time, providing an alternative means by which iron could contribute to the variability in cell free LDL oxidation. The extent of oxidation to the iron(III) state was quantified using the basic FOX assay protocol. It required 900µl of media to be adjusted to a pH between 1.7 and 1.8 with sulphuric acid, before being mixed with 50µl of 5mM xylenol orange (in 25mM H_2SO_4) and 50µl of either water or 1mM hydrogen peroxide. Samples were subsequently incubated in the dark for 30 minutes, providing adequate time for all iron(III) to react with xylenol orange and form a coloured complex with a peak absorbance at 560nm. The water blank was used when measuring iron(III) concentrations (reaction 15), while hydrogen peroxide oxidizes any iron(II) present and was therefore required for the detection of any iron in this reduced oxidation state (reaction 16 followed by reaction 15). Iron(II) levels could then be quantified as the difference between absorbances in the presence and absence of the hydrogen peroxide.

(15) Ham's F10 Fe(III) + XO + H_2O → Ham's F10 Fe(III)-XO

(16) Ham's F10 Fe(II) + H_2O_2 → Ham's F10 Fe(III) + OH^- + $^{\bullet}OH$

Despite iron(III) not being listed as a constituent of Ham's F10, high concentrations were detected in each of the four Ham's F10 media tested (Table 4.2). This contrasted with the discovery of low levels of iron(II) and results in a ratio of one iron(II) molecule for every 2.2 to 2.9 iron(III) molecules. Although Ham's F10 is formulated with 3µM ferrous sulphate, the combined iron(II) and iron(III) levels in the four media never exceeded 2.27µM. These lower than expected readings could have been caused by the formation of complexes between various components of the medium and iron in such a way that the latter was no longer available for reaction in the FOX assay. Alternatively, it may simply be due to insensitivity in the detection method. Nevertheless, mixing the Ham's F10 with Chelex 100 resin for at least four hours at 4°C did appear to remove both iron(II) and iron(III) from the medium, with significant levels no longer being detectable by the FOX assay (Table 4.2).

A series of experiments therefore investigated the effect of chelexed Ham's F10 on cell-mediated and cell free LDL oxidation after fresh supplementation with a range of ferrous sulphate concentrations (Figure 4.8). As expected, when non-chelexed Ham's F10 was supplemented with the usual 1.5µM ferrous sulphate, cell free oxidation displayed a fast rate of reaction and was no longer significantly different from the level

of cell-mediated LDL oxidation by 12 hours (Figure 4.8). Conversely, chelexed Ham's F10 supported no oxidation when supplemented with only 1.5μM ferrous sulphate (Figure 4.8a). This was observed even in the presence of THP-1 macrophages and after 24 hours of incubation. Supplementation with 2μM ferrous sulphate also prevented cell free LDL oxidation in chelexed Ham's F10 but did permit a limited degree of cell-mediated oxidation (Figure 4.8b). The addition of 2.5μM ferrous sulphate to chelexed Ham's F10 further maximized this effect (Figure 4.8c). Cell free oxidation was still prevented but cell-mediated LDL oxidation was promoted at a reasonable rate and was almost comparable to that observed when using non-chelexed Ham's F10.

For this reason, subsequent experiments tested the consistency of LDL oxidation using freshly chelexed Ham's F10 supplemented with 2.5μM ferrous sulphate. Cell free LDL oxidation was found to consistently give base-line readings under these conditions (Figure 4.9). However, the rate of cell-mediated LDL oxidation now varied dramatically from one experiment to the next. On some occasions there was an absence of any apparent oxidation, as supported by the complete retention of LDL-associated vitamin E (Figure 4.9a). At other times cell-mediated oxidation was significantly different from the cell free LDL oxidation ($p<0.001$) and proceeded, as expected, with a complete loss of vitamin E and maximal levels of protein hydroperoxides (Figure 4.9b).

Table 4.2 Iron concentrations in different bottles of Ham's F10 media.
The FOX assay was used to measure the quantity of iron(II) and iron(III) in two bottles each of Gibco BRL Ham's F10 and Sigma Ham's F10. Concentrations were determined against a standard curve that had been prepared by dissolving known concentrations of iron(II) in 25mM H_2SO_4 and incubating for 30 minutes with 50μl each of xylenol orange and 1mM hydrogen peroxide. Iron levels were also assessed in freshly chelexed Sigma Ham's F10. Results are displayed as mean ± SEM of triplicate samples.

	Fe(II) concentration (μM)	Fe(III) concentration (μM)	Total concentration (μM)	Fe(II):Fe(III) Ratio
Gibco BRL 1	0.56 ± 0.06	1.27 ± 0.04	1.83 ± 0.07	1 : 2.3
Gibco BRL 2	0.54 ± 0.06	1.19 ± 0.05	1.73 ± 0.08	1 : 2.2
Sigma 1	0.54 ± 0.05	1.50 ± 0.04	2.04 ± 0.06	1 : 2.8
Sigma 2	0.59 ± 0.06	1.68 ± 0.03	2.27 ± 0.07	1 : 2.9
Chelexed Sigma	0.00 ± 0.00	0.00 ± 0.02	0.00 ± 0.02	

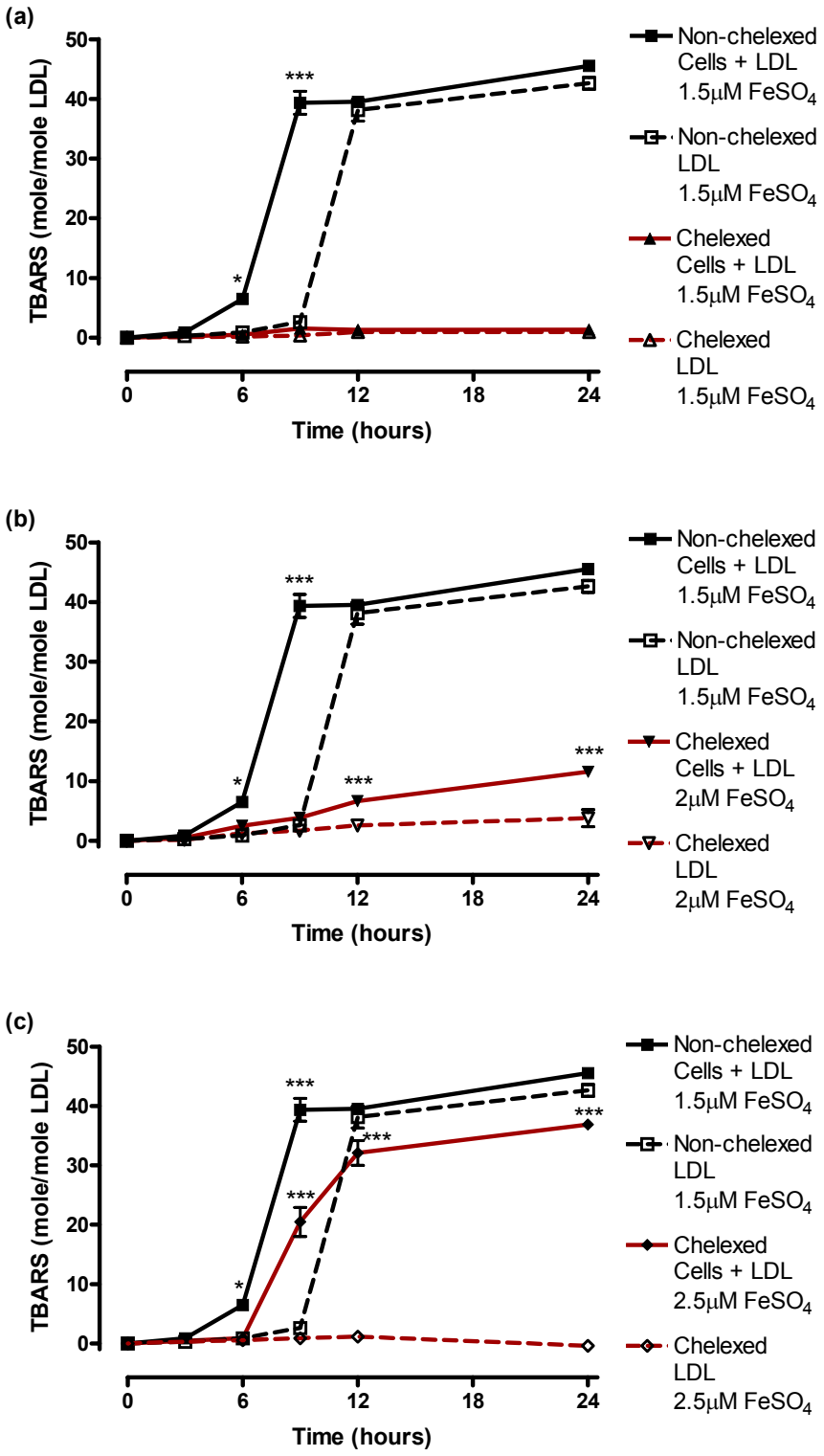


Figure 4.8 Combined effects of different iron concentrations and chelexed Ham's F10 on LDL oxidation.

LDL (0.1mg/ml) was incubated at 37°C in Ham's F10 in the presence or absence of adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml). The Ham's F10 was chelexed, contained 25mM HEPES, and was supplemented with (a) 1.5μM $FeSO_4$, (b) 2μM $FeSO_4$ and (c) 2.5μM $FeSO_4$. For comparison, each graph also includes data obtained during LDL oxidation in non-chelexed Ham's F10, supplemented with 1.5μM $FeSO_4$. All samples were analyzed at multiple time points for an increase in TBARS. Significance is indicated, at each iron concentration, between cell-containing and cell free treatments. Results are displayed as mean ± SEM of triplicate samples.

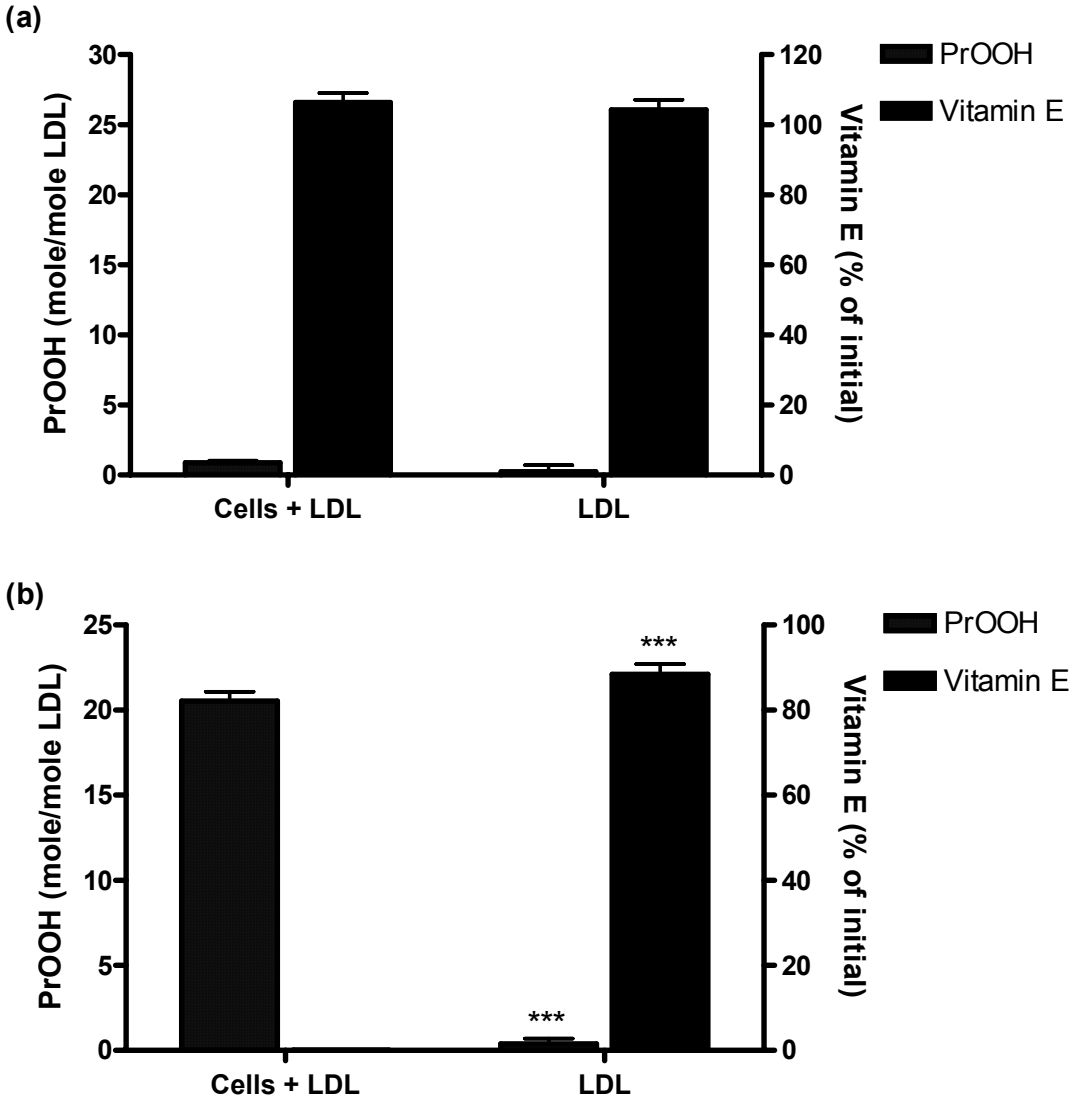


Figure 4.9 Inconsistent cell-mediated LDL oxidation using chelexed Ham's F10 and 2.5µM $FeSO_4$.

LDL (0.1mg/ml) was incubated in Ham's F10 at 37°C in the presence or absence of adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml). Ham's F10 contained 25mM HEPES and was freshly chelexed and supplemented with 2.5µM $FeSO_4$. After 24 hours, samples were analyzed for an increase in protein hydroperoxides and a loss of vitamin E. The experiments were conducted on two different occasions: (a) 15 March 2005 and (b) 29 March 2005. Significance is indicated between treatments. Results are displayed as mean ± SEM of triplicate samples.

In an attempt to gain consistency during both cell free and cell-mediated LDL oxidation, chelexed Ham's F10 was supplemented with 4.5μM ferrous sulphate and 0.01μM cupric chloride. These concentrations mimic the initial levels used before variability became a problem, representing the transition metals normally present in Ham's F10 plus the supplemental 1.5μM ferrous sulphate. Cell free LDL oxidation was still limited under these conditions, with no protein hydroperoxide formation and significant vitamin E loss ($p<0.05$) beginning only at 24 hours (Figure 4.10). By contrast, cell-mediated LDL oxidation occurred at a reasonable rate. This rate was, admittedly, slower than that previously observed when using similar concentrations of transition metals (Figure 4.1 or Figure 4.2). However, it was to be expected because the cells had been pre-incubated in Ham's F10 for 48 hours prior to the start of the experiment. This was done in conjunction with an experiment designed to investigate the effect of IFN-γ on THP-1 macrophage-mediated LDL oxidation (see section 5.25 for more details). Ham's F10 is a less nutrient-rich medium than RPMI but this pre-incubation was required to provide adequate time for IFN-γ to induce the THP-1 macrophages to synthesize 7,8-NP. A similar incubation was conducted in non-chelexed Ham's F10, supplemented with 1.5μM ferrous iron, and was also associated with a slower rate of cell-mediated LDL oxidation (data not shown).

Subsequent experiments confirmed consistency in the rate of cell-mediated LDL oxidation but indicated that some variability persisted during cell free oxidation (compare the different rates in Figure 4.10 and Figure 5.31). Nevertheless, this variability was considerably less pronounced than that experienced when using non-chelexed Ham's F10. Cell free LDL oxidation in the latter medium produced lag phases ranging anywhere from three to 24 hours. Detecting maximal levels of cell free oxidation products by 12 hours was also not uncommon. Contrary to these results in non-chelexed media, a lag phase of less than 12 hours was never observed during cell free LDL oxidation using chelexed Ham's F10. Casual observations also suggested that any cell free variation occurred less frequently than had been encountered when using non-chelexed media.

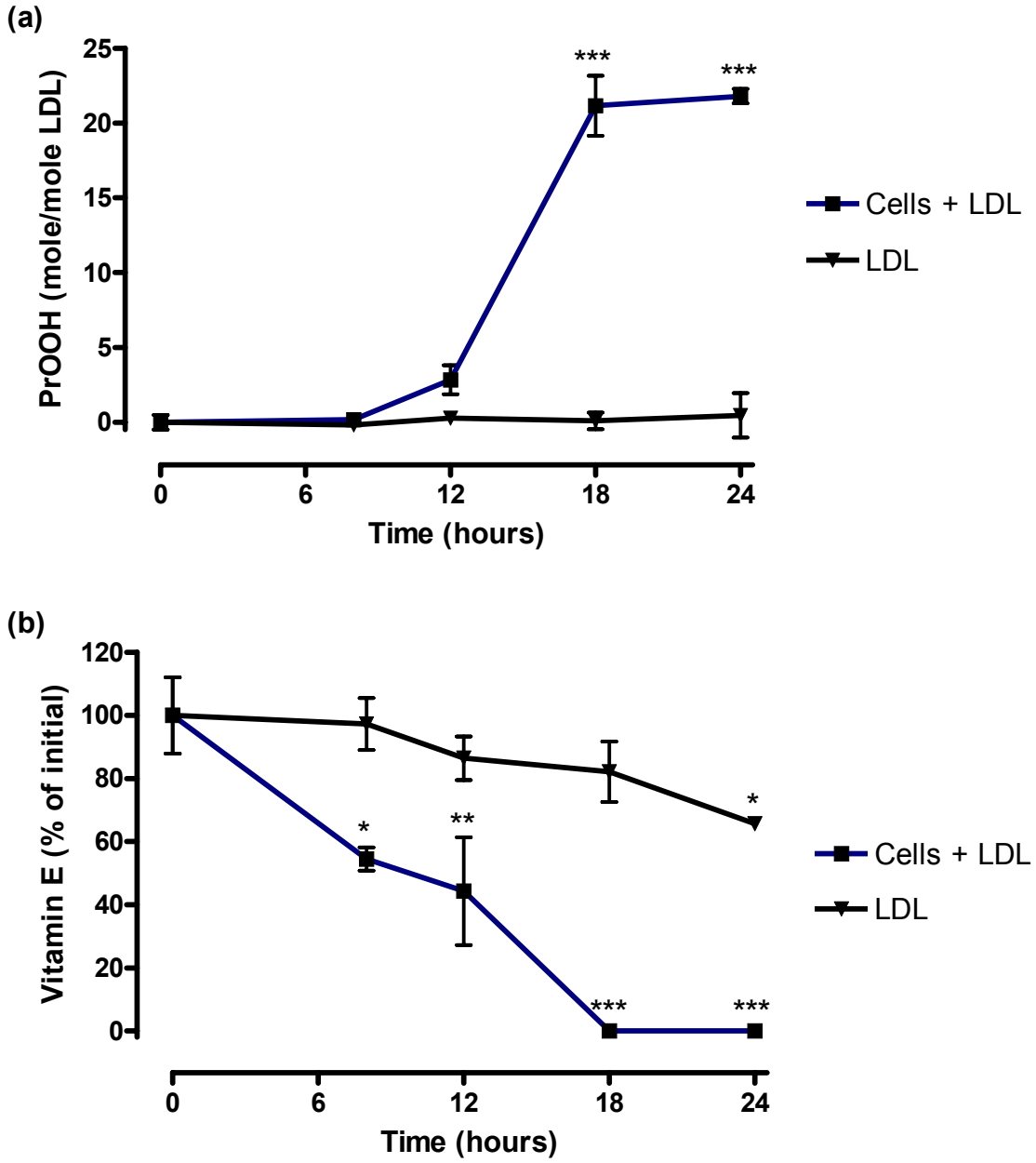


Figure 4.10 Slow cell free LDL oxidation using chelexed Ham's F10 with 4.5µM $FeSO_4$ and 0.01µM $CuCl_2$.

LDL (0.1mg/ml) was incubated in Ham's F10 at 37°C in the presence or absence of adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml). Ham's F10 contained 25mM HEPES and was freshly chelexed and supplemented with 4.5µM $FeSO_4$ and 0.01µM $CuCl_2$. Samples were analyzed at various time points for (a) an increase in protein hydroperoxides and (b) a loss of vitamin E. Significance is indicated from time zero. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

4.3 Discussion

LDL can become oxidized via multiple pathways, with the precise mechanism depending both on the LDL particle and the surrounding environment. This vast array of potential interactions and modulating factors made it difficult to identify the cause of the problem when sudden instability was observed in the rate of cell free LDL oxidation (Figure 4.1 & Figure 4.2). Despite this confusion, it soon became apparent that the variability was real. It was not an artefact of the protocols used to monitor oxidative damage because the same result was observed whether measuring protein hydroperoxides, TBARS or vitamin E levels (Figure 4.1 & Figure 4.2). Three general theories regarding the cause of this inconsistency therefore remained to be investigated:

1) Non-media related effects
2) Contaminants in the media
3) Changes in the media's chemical constituents

4.3.1 Non-Media Related Effects

LDL composition is known to be a critical determinant of the susceptibility of these particles to oxidative modification (Jessup *et al.*, 1990; Esterbauer *et al.*, 1991; Thomas & Jackson, 1991; Thomas *et al.*, 1994a; Thomas *et al.*, 1994b; Visioli *et al.*, 2000a). It was possible to minimize any interference arising from differences in LDL's composition simply by pooling LDL from multiple donors. Variation due to compositional changes also seemed unlikely given the consistent rates observed for macrophage-mediated LDL oxidation. Furthermore, large variations in the level of oxidation were observed for the four cell free treatments in Figure 4.3. This occurred despite setting up the treatments simultaneously and incubating with LDL from the same preparation.

Testing all four treatments at the same time also indicated that the problem was not related to some minor change in experimental protocol from one experiment to the next. The sources of supplemental iron and tissue culture plasticware were identical for all treatments, as were the atmospheric conditions. This latter point is of relevance because oxygen can serve as a rate limiting factor during LDL oxidation (Raveh *et al.*, 2002).

Ultimately, the source of the Ham's F10 medium provided the only point of difference between the four treatments shown in Figure 4.3.

4.3.2 Contaminants in the Media

Contaminants were most likely to enter the media either as impurities in the water or by leaching from the bottles used to store the Ham's F10. Neither option was categorically disproved, but contamination seemed unlikely considering that variability was observed whether the medium was sourced from Sigma or Gibco BRL (Figure 4.1, Figure 4.2 & Table 4.1). Not only were these media obtained from different companies, but they were stored and prepared in distinctly different ways. Ham's F10 from Gibco BRL was pre-made and supplied as a liquid in plastic containers. By contrast, Sigma medium was purchased in powder form and prepared using nanopure, ultrafiltered water. The inability to stop variable rates of cell free oxidation after preparing Sigma Ham's F10 in water sourced from an alternative filtration system provided further evidence against the effect of any impurities in the water.

4.3.3 Changes in the Media's Chemical Constituents

The variability in cell free LDL oxidation was clearly medium-dependent and caused by one or more of the chemical constituents in Ham's F10. Given that a general trend of increasing instability was observed as the Ham's F10 aged, it appeared that the component(s) may be degrading, oxidizing, reducing or reacting with other compounds over time. However, the complexity of the Ham's F10 medium made it a difficult and time-consuming process to identify and correct the cause. Initial attempts were therefore made to identify the problem without directly identifying the compounds responsible.

HEPES and riboflavin are known to synergistically generate hydrogen peroxide in the presence of light (Halliwell & Butt, 1972). Both are components of Ham's F10, but light-dependent oxidative stress reactions did not play a pivotal role in the cell free inconsistency. The storage of some Ham's F10 in darkened bottles, with minimal handling, failed to prevent cell free LDL oxidation from occurring at a rapid rate (Figure 4.3).

HEPES and phenol red were each added to Ham's F10 in an attempt to resolve the inconsistency issue through antioxidant supplementation. While HEPES is generally considered as a buffer, it can also exhibit radical scavenging activity under certain circumstances (Hicks & Gebicki, 1986; Simpson *et al.*, 1988; Ermilov *et al.*, 1999; Van Eden & Aust, 2001). Adding HEPES at the time of media preparation delayed the onset of variability, thereby stabilizing cell free oxidation for a brief time. By contrast, the

addition of 25mM HEPES to pre-made Ham's F10 immediately prior to an experiment failed to have a major impact on the extent of cell free LDL oxidation (Figure 4.3). In this experimental system, HEPES therefore appears to stabilize some component in Ham's F10 rather than exerting a direct antioxidant effect on the cell free LDL oxidation. HEPES ultimately failed to solve the problem for extended periods of time, or even consistently from one batch of medium to the next. Nevertheless, all future preparations of Ham's F10 (including those used for experiments in chapter 5) were supplemented with 25mM HEPES at the time of preparation because of the limited stabilization it did provide.

Phenol red has also been reported to exhibit antioxidant activity (Fujiwara *et al.*, 1998; Ermilov *et al.*, 1999; Dugas *et al.*, 2000; Faure *et al.*, 2004) but, despite this, phenol red was unable to efficiently scavenge radicals under the conditions of the current study (Figure 4.4). The reason for the lack of antioxidant effect is unknown but it does support a previous study of LDL oxidation in RPMI, where both the cell-mediated and cell free oxidation rates remained unchanged whether phenol red was present or absent (van Reyk *et al.*, 1999).

Having failed to resolve the instability issue either by supplementing the medium with additional antioxidant components or by varying physical parameters, the only remaining solution was to try and identify the component(s) in the medium responsible for the variability in cell free LDL oxidation rates. Ham's F10 is a complex medium, consisting of amino acids, vitamins, inorganic salts and a variety of other components (glucose, HEPES, hypoxanthine, lipoic acid, phenol red, sodium pyruvate and thymidine). One of these additional components, glucose, is known to enhance cell free LDL oxidation in the presence of transition metals (Hunt *et al.*, 1994; Kawamura *et al.*, 1994; Mowri *et al.*, 2000; Leoni *et al.*, 2002; Gallego-Nicasio *et al.*, 2003). Many of the other constituents could also potentially contribute to either the promotion or inhibition of LDL oxidation. Considering the general trend of increasing instability as the Ham's F10 aged, it seemed likely that the compound(s) responsible were somehow changing over time. Despite the complexity of Ham's F10, most of the constituents are actually relatively stable. Three notable exceptions do exist – cysteine, glutamine and ferrous ions.

Thiols can exhibit antioxidant or pro-oxidant activity, with the exact outcome largely depending on conditions in the surrounding environment. Thiols incubated in the

presence of iron, as occurs in Ham's F10, have generally been associated with a pro-oxidant activity (Parthasarathy, 1987; Graham *et al.*, 1994; Lynch & Frei, 1997; Dugas *et al.*, 2000). Wood *et al.* (1995) noted a strong positive correlation between the susceptibility of thiols to iron-mediated autoxidation and the extent of cell free LDL oxidation. Despite the detection of all medium thiols between the range of 1.8μM and 3.0μM, large variations in the extent of cell free LDL oxidation continued to be observed (Figure 4.5). The loss of 99% of the Ham's F10 cysteine is in agreement with other studies, which also commented on the relative instability of cysteine in Ham's F10 and its ultimate autoxidation to cystine (Sparrow & Olszewski, 1993; Lynch & Frei, 1997; Niu *et al.*, 2000). Cystine is unlikely to contribute to the fast rates of oxidation because previous research has indicated that cystine is unable to promote cell free oxidation on fresh LDL even in the presence of transition metals (Heinecke *et al.*, 1993a; Sparrow & Olszewski, 1993; Graham *et al.*, 1994; Santanam & Parthasarathy, 1995). Furthermore, the lack of a correlation between thiols and the extent of LDL oxidation also suggests, by extension, a lack of a correlation with cystine or any radical by-products of the autoxidation.

Glutamine has also been reported to be unstable in media (Sigma product information sheet), and many researchers supplement media with additional glutamine immediately prior to an experiment to overcome this issue (Leist *et al.*, 1996; van Reyk *et al.*, 1999; Wood & Graham, 1999; Carpenter *et al.*, 2001). Glutamine is not generally regarded as an antioxidant, but like other amino acids, could potentially serve as an alternative target of oxidative attack. This may be of particular relevance in Ham's F10 because glutamine is present at a concentration of 1mM. All other amino acid residues were added at concentrations of 200μM or less. Sanatnam & Parthasarathy (1995) noted that copper-mediated oxidation of LDL was inhibited during co-incubation with the amino acids glutamate, lysine, glycine, alanine, methionine, serine or histidine. Although glutamine was not studied in this paper, it is possible that it could have exerted similar effects and that these effects decreased in the Ham's F10 over time as the glutamine degraded. This theory was disproved when it became apparent that supplementation with additional fresh glutamine failed to change the extent of cell free LDL oxidation (Figure 4.6).

Metal ion dependent oxidative modification of LDL has been extensively studied, but the exact mechanism of action still remains to be elucidated. Nevertheless, it is clear that copper and iron promote LDL oxidation by slightly different mechanisms (Lynch

& Frei, 1993; Tribble *et al.*, 1996) and that redox chemistry plays a pivotal role in the oxidative process (Tang *et al.*, 2000).

Copper is a more potent inducer of LDL oxidation than iron (Lynch & Frei, 1993; Kritharides *et al.*, 1995a; Lynch & Frei, 1995) but is present in the Ham's F10 medium at a concentration of only 0.01μM. Redox cycling provides a mechanism for minimizing the limitations of this low concentration, with both LDL (Lynch & Frei, 1995) and ferrous ions (Kritharides *et al.*, 1995a) reported to reduce copper to its redox active cuprous ion state. The speed of the reduction mediated by LDL (Lynch & Frei, 1995) suggests that any change in the copper redox status during Ham's F10 storage is unlikely to directly account for the variability associated with the cell free LDL oxidations. Copper reduction may do so indirectly, however, by enhancing the oxidation of iron over time.

Iron is the predominant transition metal in Ham's F10, with an initial formulation of 3μM ferrous sulphate and an additional 1.5μM included immediately prior to an experiment. Iron chemistry can be quite complex and is further complicated by the tendency of any ferrous sulphate present in Ham's F10 to autoxidize to the iron(III) state (Wood & Graham, 1995; Dugas *et al.*, 2000). Changes in the Ham's F10 iron redox state were also observed in the current study (Table 4.2) and are likely to be of consequence to the medium problem. Iron-mediated LDL oxidation has been reported to occur at a significant rate only when both iron(II) and iron(III) are present. Some studies suggest a requirement for an, as yet unidentified, iron(II)-iron(III) complex (Minotti & Aust, 1987; Tang *et al.*, 1997; Welch *et al.*, 2002b). Others have argued against this and, instead, suggested that all observations could be explained by the balance between iron(II)'s decomposition of pre-existing lipid hydroperoxides (reaction 17) and its scavenging of the resulting lipid alkoxyl and peroxyl radicals (reactions 18 & 19) (Tang *et al.*, 2000). A further decomposition reaction, mediated by iron(III), is described in reaction 20 but may be of little relevance *in vivo* because it is expected to proceed slowly relative to reaction 17. Based on the above theory, the lipid peroxidation chain reaction (reactions 21 & 22) would begin only once iron(II) has been oxidized to such an extent that it can no longer effectively compete with lipid for the alkoxyl/peroxyl radicals (reactions 18 & 19).

(17) $Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\bullet} + OH^{-}$

(18) $Fe^{2+} + LO^{\bullet} + H^{+} \rightarrow Fe^{3+} + LOH$

(19) $Fe^{2+} + LOO^{\bullet} + H^{+} \rightarrow Fe^{3+} + LOOH$

(20) $Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO^{\bullet} + H^{+}$

(21) $LO^{\bullet}/LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOH/LOOH$

(22) $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

Whatever the mechanism, changes in the relative amounts of iron(II), iron(III) and lipid obviously have the potential to modulate the rate of cell free LDL oxidation. Differences in these parameters from one batch of medium to the next could account for the variable rates observed both over time and between bottles of media. The lipid concentration was kept constant throughout all experiments, while altering the iron(II) to iron(III) ratio by supplementation did not stabilize the rate of cell free LDL oxidation (Figure 4.1, Figure 4.2 & Figure 4.7). A more comprehensive means of modulating iron levels, and the corresponding redox states, involved chelexing the Ham's F10 and then standardising the medium by adding a known concentration of fresh ferrous sulphate immediately prior to an experiment.

The use of Chelex certainly removed all iron detectable by the FOX assay (Table 4.2) and, accordingly, chelexed medium was not permissive for either cell-mediated or cell free LDL oxidation even after resupplementation with 1.5μM iron (Figure 4.8). Increasing the iron concentration to 2.5μM maintained cell free LDL oxidation at consistently minimal rates but resulted in sudden variability for cell-mediated oxidation (Figure 4.9). Macrophages can exhibit either antioxidant or pro-oxidant activity towards LDL, with the net effect depending on the exact conditions of study (Baoutina *et al.*, 2001a). As a result, a 2.5μM iron concentration may have represented a threshold value under the current experimental conditions. Depending on the metabolic state of the THP-1s at the time of PMA-induced conversion to macrophages, the cells may have been either more or less successful at counteracting the pro-oxidant effects of this iron. One potential mechanism involves cell-dependent sequestration of the iron (van Reyk *et al.*, 1999), with successful sequestration inhibiting LDL oxidation. Failure to sequester sufficient amounts would permit the competing reaction, macrophage-mediated reduction of the remaining iron and subsequent LDL oxidation. An increased iron concentration should therefore consistently overwhelm the cell's metal sequestering activity (and all other antioxidant activity), leading to stable rates of cell-mediated LDL oxidation. This was, indeed, observed once the chelexed Ham's F10 was supplemented with 4.5μM ferrous sulphate and 0.01μM copper chloride (Figure 4.10).

Standardising the medium in this way also ensured minimal, and relatively consistent, rates of cell free LDL oxidation from one experiment to the next. The overall

iron(II) concentration, and its rate of autoxidation, are therefore critical in accounting for the variable rates of cell free LDL oxidation observed both over time and between bottles of media.

Throughout this entire study, the relative stability of cell-mediated LDL oxidation provided a direct contrast to the inconsistent rates of cell free LDL oxidation. Such a discrepancy can also be explained by the iron autoxidation theory because cells possess an inherent ability to reduce iron to the iron(II) state (Garner *et al.*, 1997a; Baoutina *et al.*, 2001b). This, in turn, minimizes the consequences of any iron autoxidation that may or may not have occurred in the medium during storage.

Finally, the apparent ability of HEPES to delay the onset of variability can now be accounted for by the fact that iron autoxidation is significantly slower in HEPES buffer than in the phosphate and bicarbonate buffers that provide buffering capacity to Ham's F10 lacking HEPES (Welch *et al.*, 2002a).

4.3.4 Summary

Inconsistency in the rate of cell free LDL oxidation was quickly attributed to some unidentified variable in the Ham's F10 medium. The addition of 25mM HEPES provided a degree of stabilization, but the problem could be more comprehensively resolved by completely removing all transition metals in the Ham's F10 via chelexing. Immediately prior to an experiment, the medium could then be standardised by resupplementing with a known concentration of fresh transition metals (4.5μM ferrous sulphate and 0.01μM cupric chloride).

The requirement for freshly prepared transition metal solutions indicates that the variable rates of cell free LDL oxidation were caused by iron autoxidation during storage of the non-chelexed media. As HEPES is reported to stabilize ferrous ions, and therefore slow their rate of autoxidation (Welch *et al.*, 2002a), this theory also accounts for the stabilizing effect of HEPES supplementation.

5 7,8-NP and Macrophage-Mediated LDL Oxidation

5.1 Introduction

Macrophage-mediated oxidation of LDL is postulated to play a critical role in the development of the atherosclerosis. Until recently, however, most attention has focussed on lipid peroxidation, antioxidant depletion and/or late stage protein modification. Early peroxidation of the apoB100 protein can be just as critical and has, in fact, been confirmed under cell free conditions (Gieseg *et al.*, 2003). Preliminary research arising from this PhD thesis was also included in the Gieseg *et al.* (2003) paper and demonstrates the formation of protein hydroperoxides on LDL in the presence of THP-1 macrophages (Figure 5, re-printed in Appendix II). Chapter 5 will therefore extend this initial finding by further monitoring the kinetics of macrophage-mediated protein hydroperoxide formation and correlating these kinetics to a range of other LDL oxidation markers.

The generation of protein hydroperoxides is of particular significance because this oxidation product is not inert. Protein hydroperoxide formation on bovine serum albumin (BSA) has been associated with peroxidation chain reactions (Neuzil *et al.*, 1993) and it seems reasonable to expect that such reactions may also occur on the apoB100 of LDL. Once formed, these protein hydroperoxides also possess sufficient reactivity to promote additional damage. Their decomposition to reactive free radicals appears to be promoted by both metal-mediated (Davies *et al.*, 1995; Gebicki *et al.*, 1995) and cell-mediated (Headlam & Davies, 2003) processes. Such decomposition is known to enhance the oxidation of nucleotides (Luxford *et al.*, 1999), formation of protein-DNA cross-links (Gebicki & Gebicki, 1999) and inactivation of various oxidized enzymes (Hampton *et al.*, 2002; Morgan *et al.*, 2002). Protein fragmentation has also been attributed to hydroperoxide degradation, specifically when the hydroperoxide is located at the α-carbon position of the peptide backbone (Davies, 1996a). Additionally, protein hydroperoxides can reduce the surrounding environment's antioxidant capacity by consuming thiols (Soszynski *et al.*, 1996) and antioxidants like GSH and ascorbate (Simpson *et al.*, 1992; Fu *et al.*, 1995).

Although protein hydroperoxides are reactive, they also possess a degree of stability (Simpson *et al.*, 1992). This contrasts with the short-lived, initiating free radicals and enables diffusion some distance from their site of formation. As a consequence, protein hydroperoxides may contribute to both localized and widespread oxidative damage, and their generation could have critical implications in atherogenesis.

Inhibiting protein hydroperoxide formation clearly represents an important area of research. Direct cellular defence against protein hydroperoxides appears limited as peroxidases are generally inefficient at degrading protein hydroperoxides, particularly when those hydroperoxides are associated with larger proteins (Gebicki *et al.*, 2002). By contrast, the macrophage-synthesized pteridine, 7,8-NP, has been shown to be a potent inhibitor of protein hydroperoxide formation during studies exposing U937 cells and bovine serum albumin to a range of oxidative insults (Duggan *et al.*, 2001; Duggan *et al.*, 2002; Gieseg *et al.*, 2003). 7,8-NP has also been observed to inhibit LDL oxidation (Gieseg *et al.*, 1995; Gieseg & Cato, 2003; Gieseg *et al.*, 2003). Protection against lipid peroxidation was noted whether the LDL was oxidized by AAPH, copper ions or THP-1 monocytes and THP-1 macrophages. Protection against protein peroxidation was studied, and confirmed, only under the cell free conditions.

The current research will therefore complement the above work by investigating 7,8-NP's efficacy, and its mode of action, in protecting the LDL particle from macrophage-mediated oxidation. Specific emphasis will be placed on 7,8-NP's inhibition of protein hydroperoxide formation. 7,8-NP will be supplied both exogenously and via *de novo* synthesis, upon stimulation of macrophages with IFN-γ. Several studies have previously used mouse cells to investigate the effect of IFN-γ on macrophage-mediated LDL oxidation (Jessup *et al.*, 1992; Jessup & Dean, 1993; Fong *et al.*, 1994; Niu *et al.*, 2000). Although LDL oxidation was inhibited in all these mouse studies, the results are not applicable to human macrophages. Protection was attributed to nitric oxide but, in human macrophages, IFN-γ induces 7,8-NP synthesis rather than nitric oxide production (Werner *et al.*, 1990). Thus, differentiated THP-1s will be used as one source of macrophages for the current research. As cell lines are not completely representative of cells in the body, an additional investigation will use primary HMDMs to more conclusively indicate whether 7,8-NP could inhibit LDL oxidation *in vivo*.

5.2 Results

5.2.1 Protein Hydroperoxide Formation on LDL During Oxidation Mediated by THP-1 Macrophages

Conjugated dienes on LDL can be rapidly measured at 234nm without any need for prior lipid extraction, thereby providing a convenient means of monitoring lipid peroxidation. Although the Ham's F10 culture medium contains components that also absorb at 234nm (Esterbauer *et al.*, 1992), Beer's Law still applies up to an absorbance value of at least 2.15 units (data not shown). The formation of conjugated dienes could therefore be monitored when investigating the iron(II) concentration required for optimal cell-mediated LDL oxidation. TBARS provided an additional indication of lipid peroxidation while protein hydroperoxides highlighted the extent of protein oxidation. These initial studies were conducted in non-chelexed Ham's F10 and, as such, contained a transition metal baseline of 3μM ferrous sulphate and 0.01μM cupric chloride.

A lack of additional iron(II) resulted in minimal levels of both cell free and THP-1 macrophage-mediated LDL oxidation, whether monitored via protein hydroperoxides, TBARS or conjugated dienes (Figure 5.1). By contrast, supplementing the Ham's F10 with 1.5μM (Figure 5.2) or 3μM (Figure 5.3) iron(II) yielded clear kinetic data. LDL oxidized by THP-1 macrophages in the 1.5μM iron(II)-supplemented medium exhibited a longer lag phase than LDL oxidized in the corresponding 3μM iron(II)-supplemented medium. This led to peaks or plateaux in concentration for all three assayed oxidation parameters at nine hours rather than the six hours observed using the 3μM iron(II)-supplemented treatment. It also ensured that the decomposition phase was strongly detected only at the higher iron concentration during these 12 hour time courses. Such a limitation was deemed to be of little relevance to the current research because antioxidant efficacy studies generally centre on the lag and propagation phases rather than advanced stages of decomposition. Of more importance was the confirmation that the peak in protein hydroperoxides corresponded to a concentration between approximately 30-40 moles/mole LDL. This concentration was attained irrespective of supplementing with 1.5μM or 3μM iron(II). Furthermore, a comparison of iron treatments indicated that the difference between cell-mediated and cell-free LDL oxidation levels was more pronounced after the addition of 1.5μM iron(II). Future experiments in non-chelexed Ham's F10 were therefore conducted by supplementing the medium with 1.5μM iron(II).

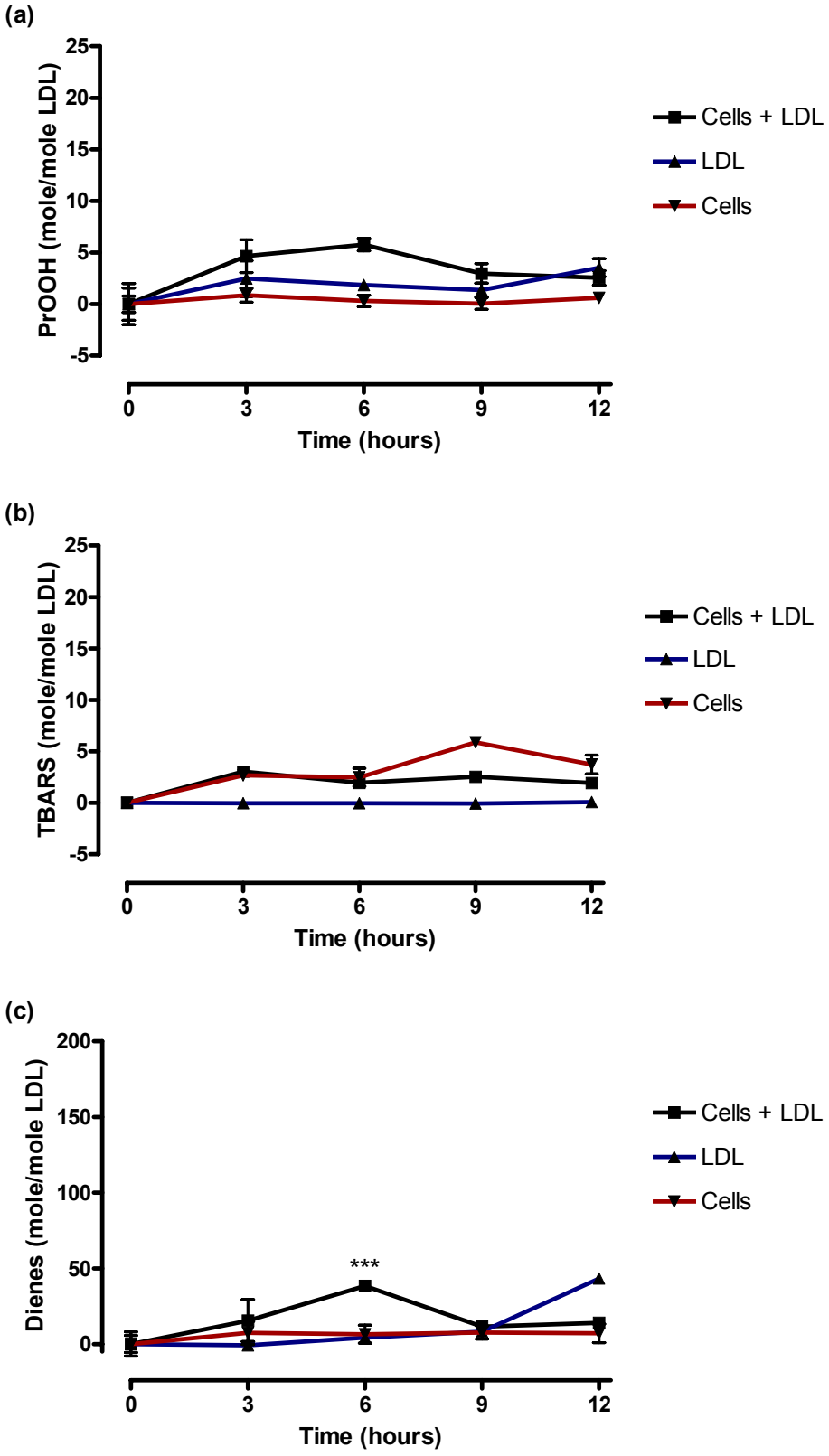


Figure 5.1 Effect of no iron supplementation on THP-1 macrophage-mediated LDL oxidation.

Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C with 0.1mg/ml LDL in Ham's F10 in the absence of any iron supplementation. Controls included cells only and LDL only. Samples were analyzed at various time points for an increase in (a) protein hydroperoxides (PrOOH), (b) TBARS and (c) conjugated dienes. Significance is indicated between LDL treatments. Results are displayed as mean ± SEM of triplicate samples.

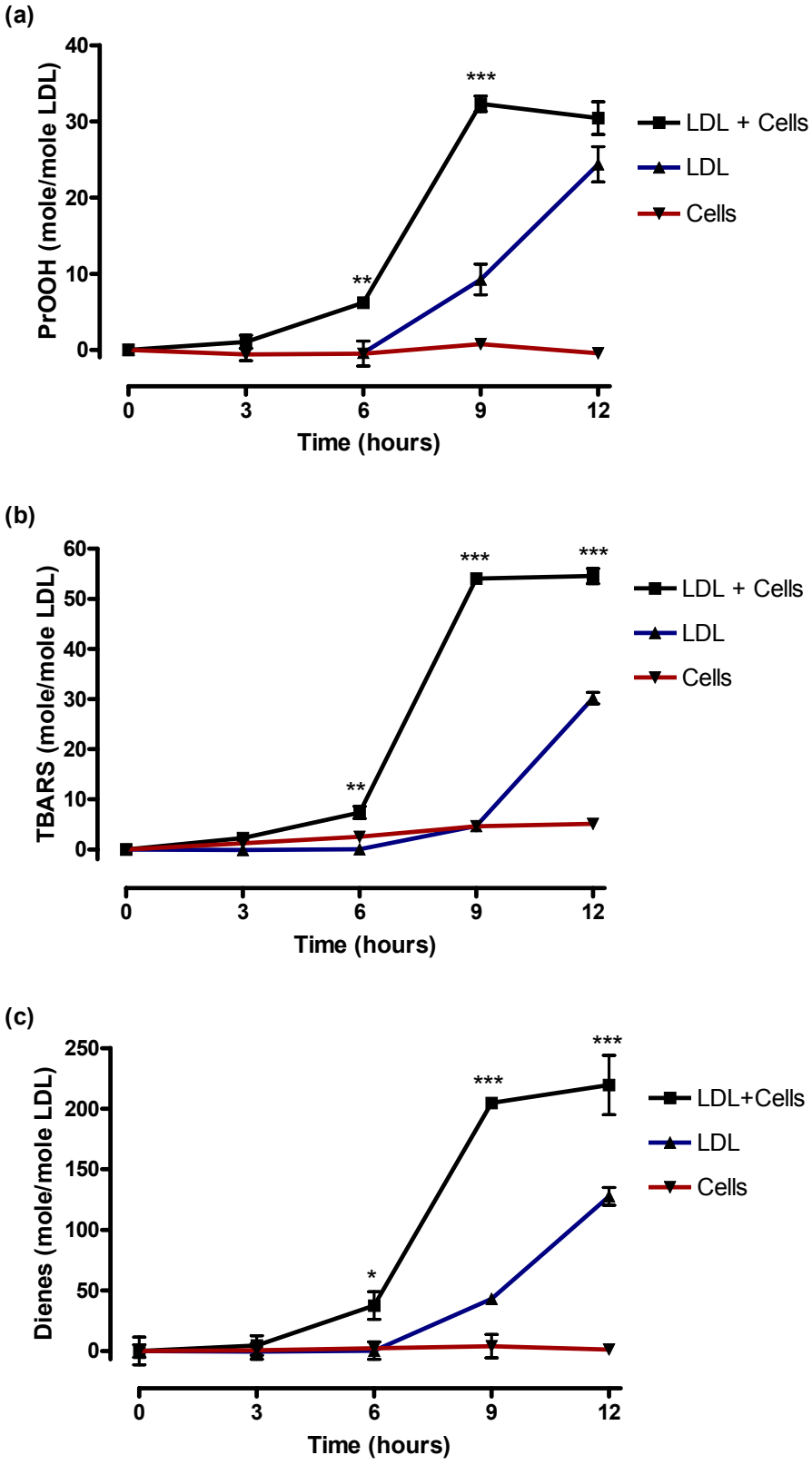


Figure 5.2 Effect of supplementation with 1.5µM iron on THP-1 macrophage-mediated LDL oxidation.

Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C with 0.1mg/ml LDL in Ham's F10 supplemented with 1.5µM $FeSO_4$. Controls included cells only and LDL only. Samples were analyzed at various time points for an increase in (a) protein hydroperoxides, (b) TBARS and (c) conjugated dienes. Significance is indicated between LDL treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

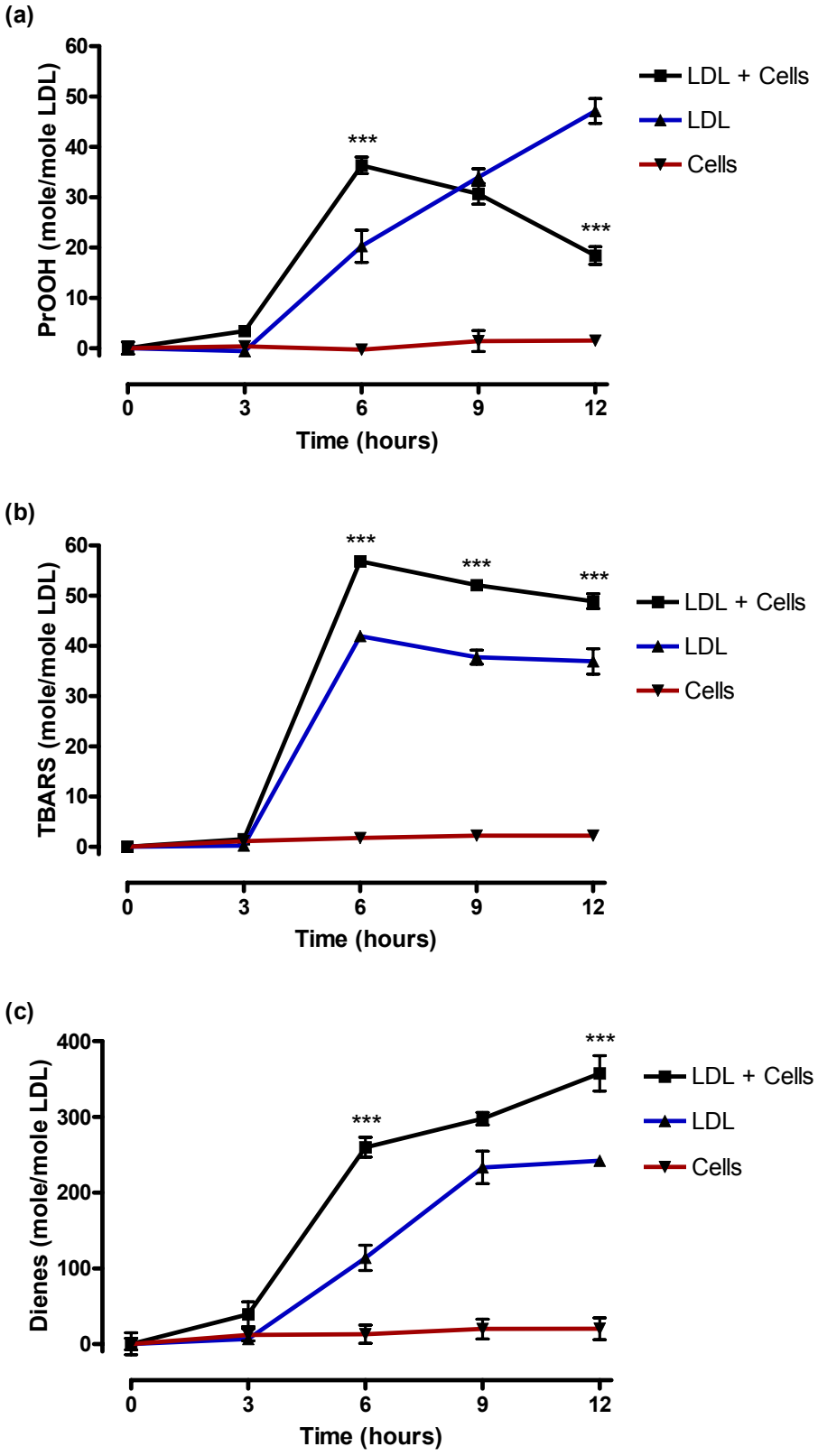


Figure 5.3 Effect of supplementation with 3µM iron on THP-1 macrophage-mediated LDL oxidation.

Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C with 0.1mg/ml LDL in Ham's F10 supplemented with 3µM $FeSO_4$. Controls included cells only and LDL only. Samples were analyzed at various time points for an increase in (a) protein hydroperoxides, (b) TBARS and (c) conjugated dienes. Significance is indicated between LDL treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

Supplementing non-chelexed Ham's F10 with 1.5µM iron(II) also promoted the loss of LDL's vitamin E in a time-dependent manner (Figure 5.4). In the presence of cells, this resulted in the complete consumption of vitamin E within six hours. Cell free oxidation was significantly slower, with 100% loss of the antioxidant only by 12 hours.

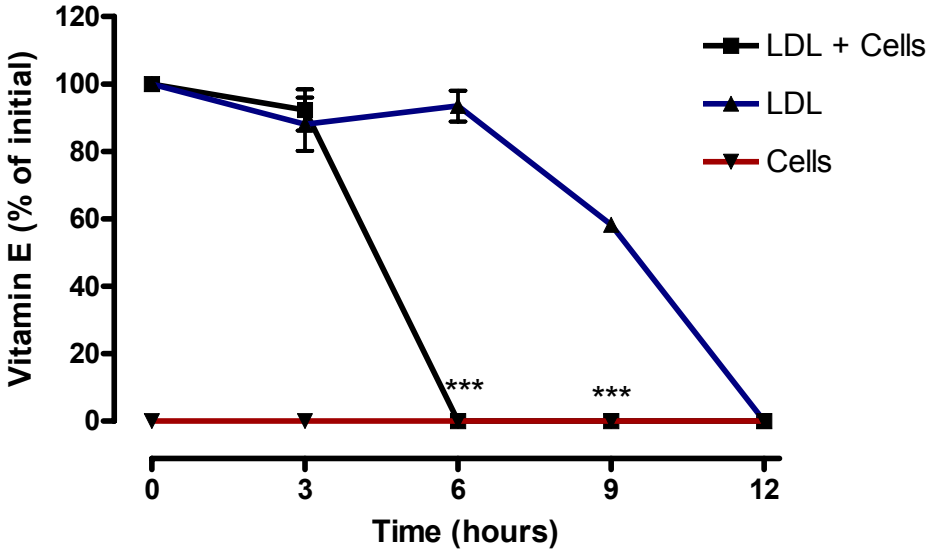


Figure 5.4 Loss of vitamin E during THP-1 macrophage-mediated LDL oxidation. Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C with 0.1mg/ml LDL in Ham's F10 supplemented with 1.5µM $FeSO_4$. Controls included cells only and LDL only, and all samples were analyzed at various time points for the loss of vitamin E. Significance is indicated between LDL treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

In an attempt to complement the protein hydroperoxide data, PB-DOPA and dityrosine were chosen to serve as additional markers of apoB100 modification and, more specifically, of tyrosine oxidation. However, neither was identified as a major product during the oxidative modification of LDL (Figure 5.5). Dityrosine was undetectable during the entire 12 hour time course, whether monitored in the presence or absence of THP-1 macrophages. PB-DOPA was measurable, but levels remained low and no significant difference between cell free and cell-mediated LDL oxidation was noted at any time point. The plateau attained after 12 hours, by both the cell free and cell-containing treatments, resulted in PB-DOPA concentrations of less than 0.3 moles/mole of LDL.

As expected, dityrosine and PB-DOPA remained undetectable in supernatant isolated from the cell only controls over the 12 hour incubation period (Figure 5.5). This finding agrees with the lack of change in the cell only control when monitoring protein hydroperoxides, conjugated dienes, TBARS and vitamin E (Figure 5.2 & Figure 5.4).

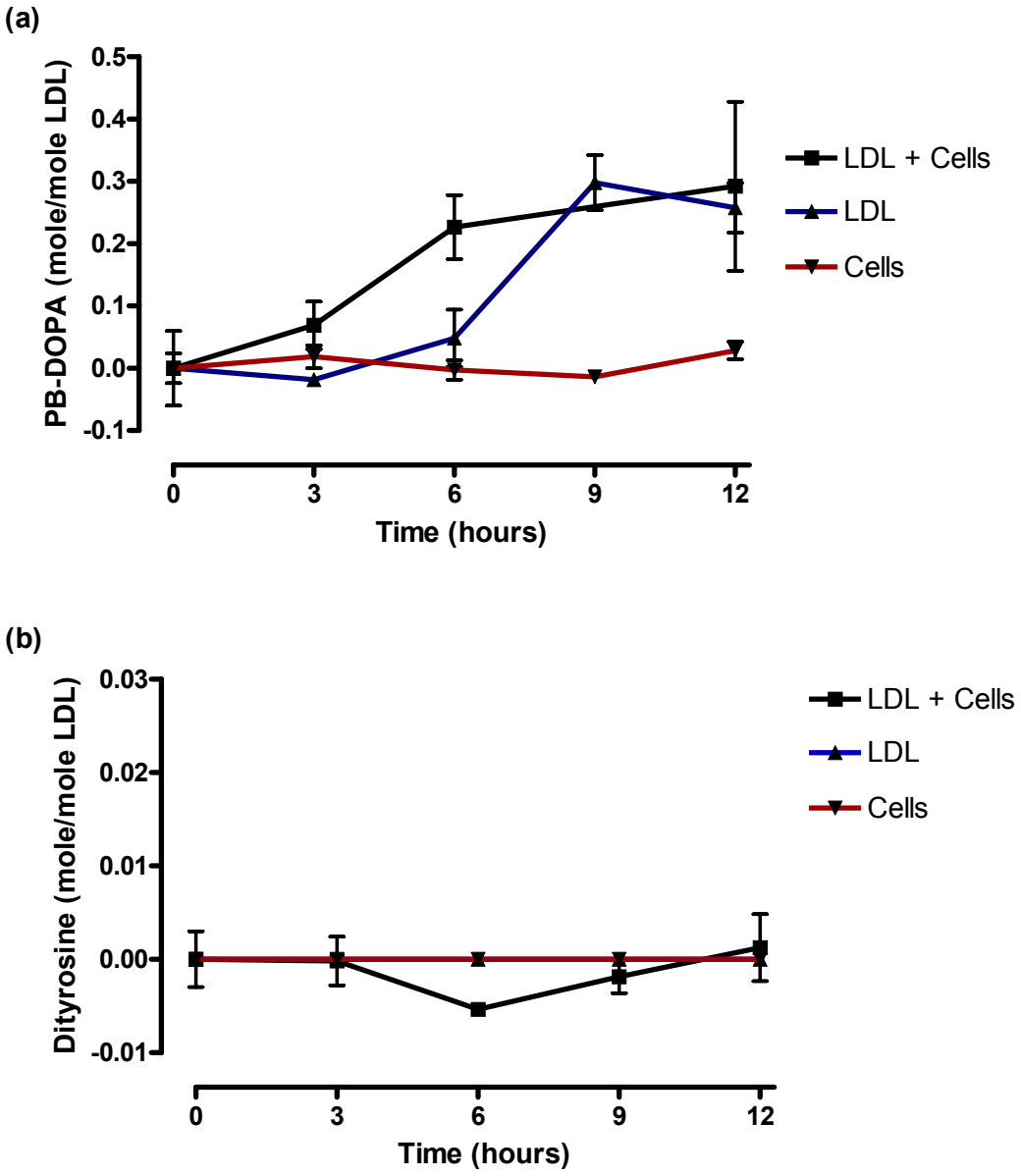
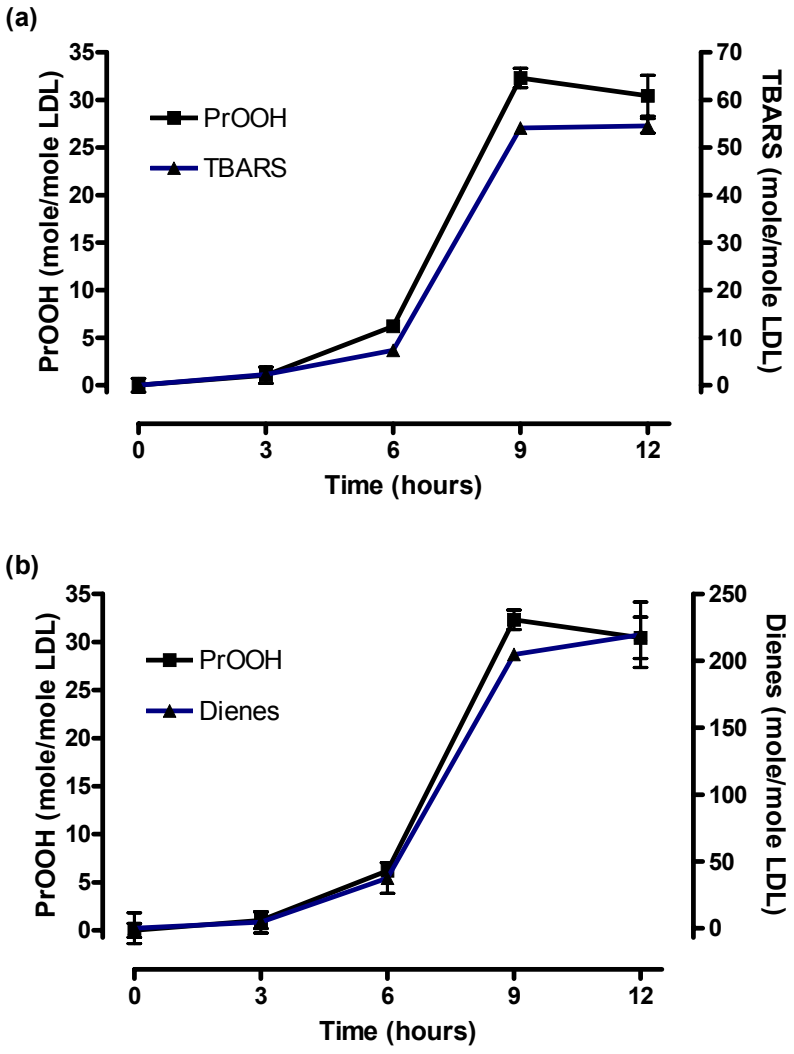


Figure 5.5 THP-1 macrophage-mediated oxidation of tyrosine residues on LDL. Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C with 0.1mg/ml LDL in Ham's F10 supplemented with 1.5µM $FeSO_4$. Controls included cells only and LDL only. Samples were analyzed at various time points for an increase in (a) PB-DOPA and (b) dityrosine. ANOVA analysis revealed no statistical significance between LDL treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

The parameters of cell-mediated LDL oxidation described above were subsequently compared to the kinetics of protein hydroperoxide formation and analyzed for any significant correlations (Figure 5.6). Specifically, protein peroxidation was shown to strongly and positively correlate with lipid peroxidation. This was observed whether monitoring TBARS (r^2=0.99) or conjugated diene (r^2=0.99) formation and it ensured that the protein hydroperoxide lag, propagation and decomposition phases paralleled the

corresponding TBARS and conjugated diene phases. While not as strong as the protein peroxidation/lipid peroxidation relationship, protein hydroperoxides were also observed to correlate with PB-DOPA (r^2=0.76) and protein carbonyls (r^2=0.65). The rate of protein hydroperoxide formation occurred faster in Figure 5.6d than previously observed. It is thought to be caused by an unexpected increase in the oxidation potency of THP-1s over time (possibly due to the creation of an unidentified mutation after numerous cell passages) and does not negate the existence of the carbonyl/protein hydroperoxide correlation.

A relationship was also confirmed between protein hydroperoxide formation and vitamin E loss, with the protein peroxidation lag phase ending and propagation beginning only once vitamin E was completely consumed (Figure 5.6e).



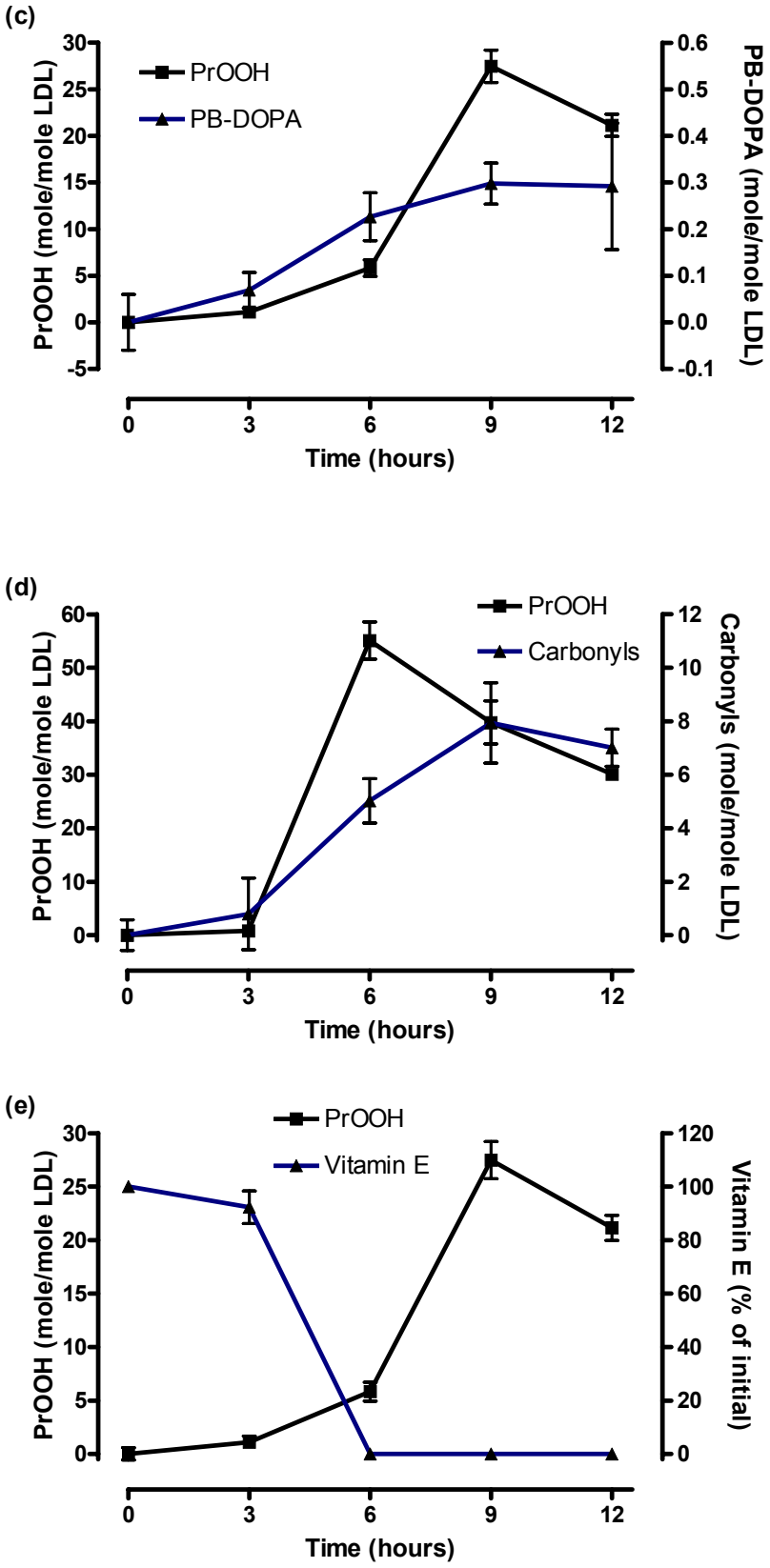


Figure 5.6 Correlations with protein hydroperoxide formation during THP-1 macrophage-mediated LDL oxidation.

Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C with 0.1mg/ml LDL in Ham's F10 supplemented with 1.5μM $FeSO_4$. Samples were analyzed at various time points for an increase in protein hydroperoxides and (a) TBARS, (b) conjugated dienes, (c) PB-DOPA, (d) carbonyls and (e) a loss of vitamin E. The data is paired and from five separate experiments.

5.2.2 Protein Hydroperoxides and THP-1 Macrophage-Mediated BSA Oxidation

A previous study had suggested that, under cell free conditions, radical intermediates of lipid peroxidation were required to initiate the formation of protein hydroperoxides on LDL (Gieseg *et al.*, 2003). Thus, although protein hydroperoxides were produced in significant quantities on LDL during macrophage-mediated oxidation, it was unclear whether pure proteins would be just as susceptible to cell-mediated peroxidation. This was investigated by incubating THP-1 macrophages with a pure protein, BSA, under conditions identical to those described for the LDL oxidation experiments. Despite testing BSA concentrations between 0.1mg/ml and 5mg/ml, the presence of $1x10^5$/ml THP-1 macrophages failed to elevate protein hydroperoxide levels above cell free controls after 12 hours (Figure 5.7).

It remained possible that the cells were oxidizing the BSA but that any protein hydroperoxides were simply degraded by the end of the 12 hour incubation period. BSA, at 0.1mg/ml (Figure 5.8) and 5mg/ml (Figure 5.9), was incubated under the same conditions as above but samples were instead collected at multiple time points. Once again, protein hydroperoxide formation remained insignificant and this was observed for both cell-mediated and cell free conditions at all time points tested.

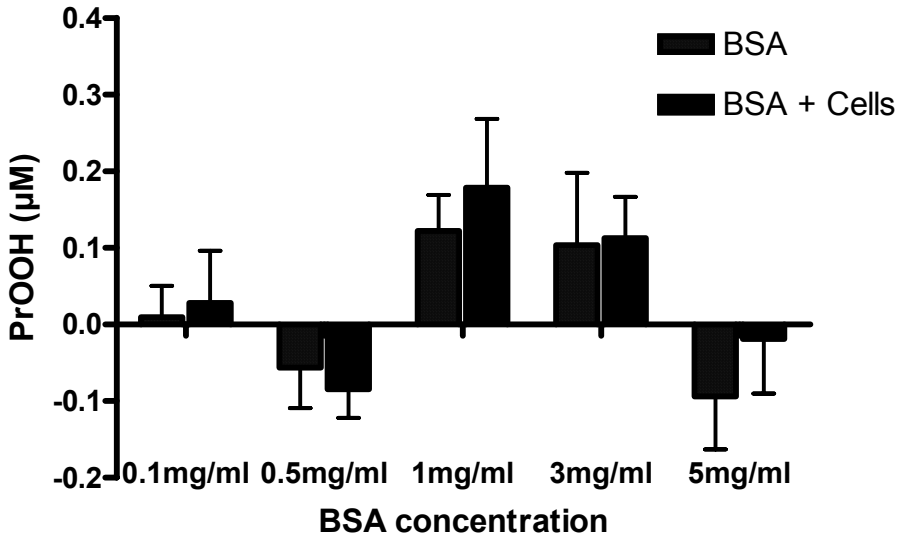


Figure 5.7 THP-1 macrophage-mediated protein hydroperoxide formation on BSA.

Various concentrations of BSA (0.1, 0.5, 1, 3 and 5mg/ml) were incubated at 37°C in the presence or absence of adherent THP-1 macrophage-like cells ($1x10^5$/ml) in Ham's F10 supplemented with 1.5µM $FeSO_4$. After 12 hours, samples were analyzed for an increase in protein hydroperoxides by the FOX-1 assay. ANOVA analysis revealed no statistical significance between BSA treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of two separate experiments.

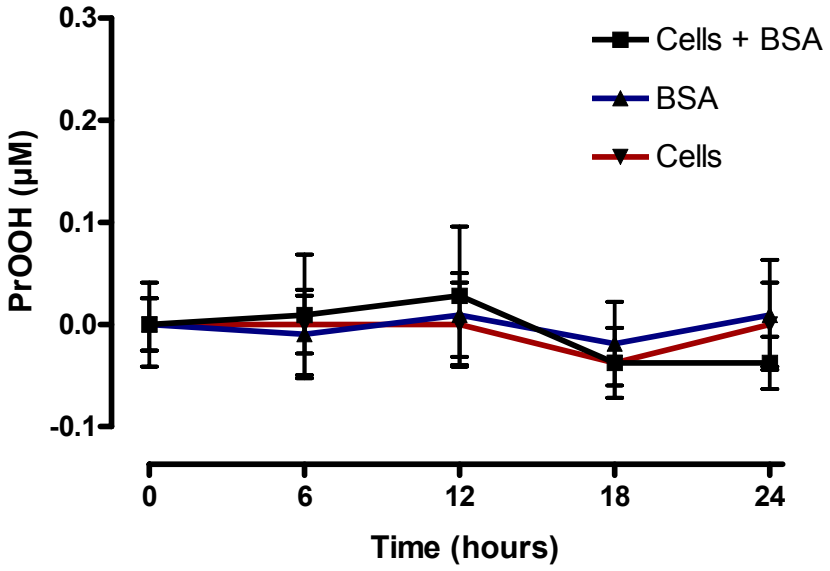


Figure 5.8 Time course of THP-1 macrophage-mediated protein hydroperoxide formation on 0.1mg/ml BSA.
Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C with 0.1mg/ml BSA in Ham's F10 supplemented with 1.5µM $FeSO_4$. Controls included cells only and BSA only. Samples were analyzed at various time points for an increase in protein hydroperoxides by the FOX-1 assay. ANOVA analysis revealed no statistical significance between LDL treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of two separate experiments.

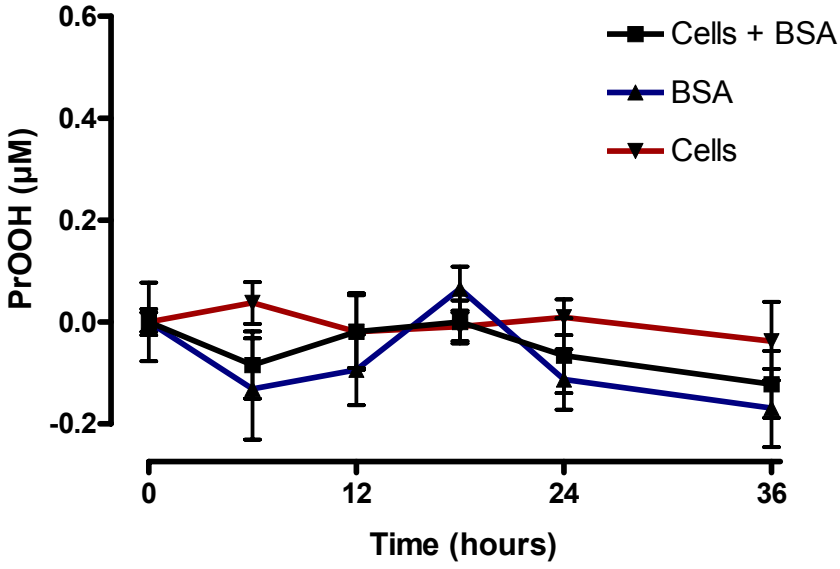


Figure 5.9 Time course of THP-1 macrophage-mediated protein hydroperoxide formation on 5mg/ml BSA.
Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C with 5mg/ml BSA in Ham's F10 supplemented with 1.5µM $FeSO_4$. Controls included cells only and BSA only. Samples were analyzed at various time points for an increase in protein hydroperoxides by the FOX-1 assay. ANOVA analysis revealed no statistical significance between LDL treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of two separate experiments.

5.2.3 7,8-NP Inhibits Protein Hydroperoxide Formation During THP-1 Macrophage-Mediated LDL Oxidation

Like Ham's F10, 7,8-NP also interferes at the 234nm wavelength (data not shown). The combined interference from Ham's F10 and 7,8-NP ultimately made reliable collection of conjugated dienes data impossible. Further complicating matters was the likely impact of 7,8-NP oxidation on the extent of interference. As a result, TBARS rather than conjugated dienes became the preferred marker of lipid peroxidation in the presence of 7,8-NP. Vitamin E and protein hydroperoxides were chosen as additional indicators of oxidation, thereby ensuring a comprehensive understanding of 7,8-NP's impact on the entire LDL particle during THP-1 macrophage-mediated LDL oxidation.

Gieseg & Cato (2003) noted that protection against cell-mediated LDL oxidation was consistent only when 7,8-NP was pre-incubated with the cells. Consequently, a five minute pre-incubation of THP-1 macrophages in Ham's F10, freshly supplemented with 1.5μM iron(II), was followed by a further five minute pre-incubation with various concentrations of 7,8-NP. Samples were then incubated in the presence of 0.1mg/ml LDL at 37°C. A nine hour incubation was chosen because it had previously been shown to represent the end of the propagation phase and, as such, a time of peak protein hydroperoxide and TBARS concentrations (Figure 5.2). Under these conditions, 7,8-NP was found to protect LDL from THP-1 macrophage-mediated protein hydroperoxide formation in a concentration-dependent manner (Figure 5.10). A similar pattern of inhibition was observed for TBARS formation and vitamin E loss. Thus, 30μM 7,8-NP afforded almost complete protection against the formation of both protein hydroperoxides and TBARS while concurrently permitting the retention of significant quantities of vitamin E.

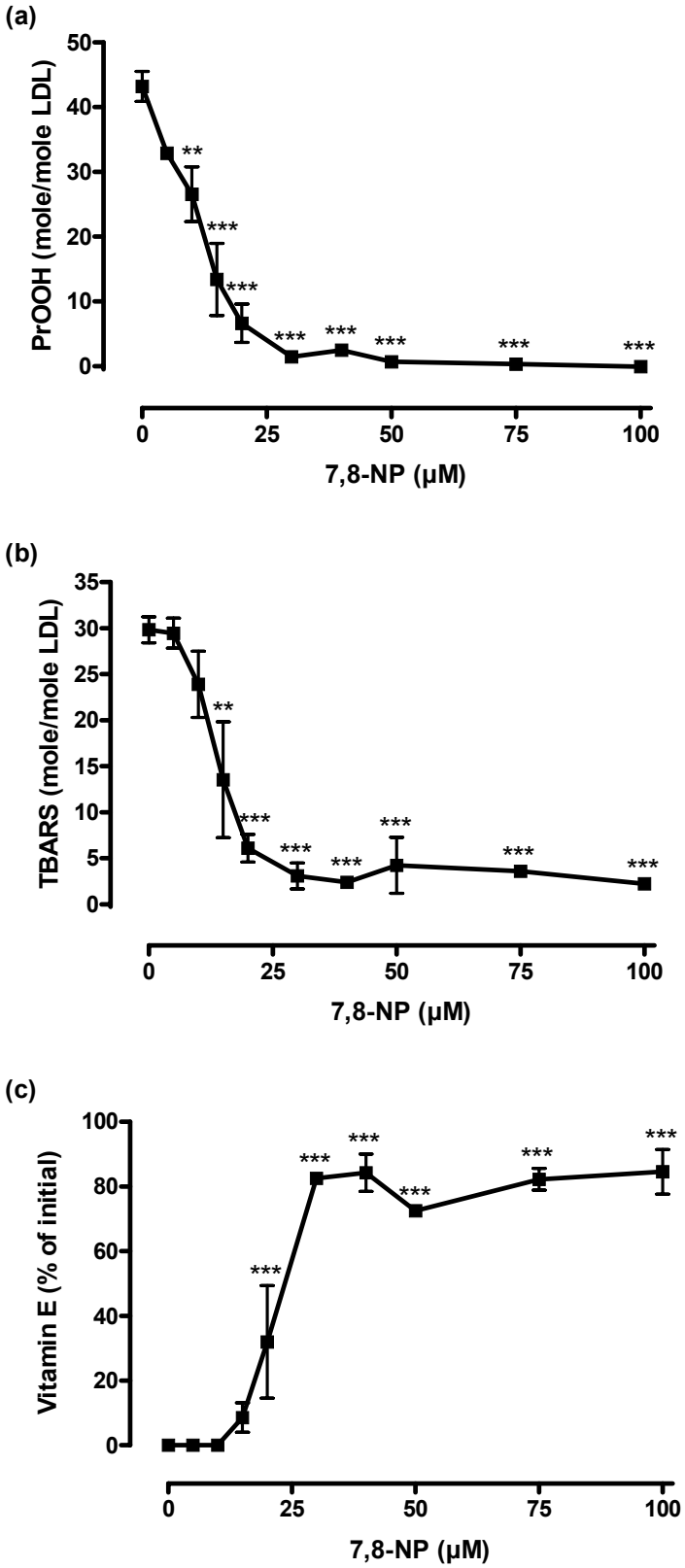


Figure 5.10 7,8-NP inhibits THP-1 macrophage-mediated LDL oxidation in a concentration-dependent manner.

Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C, with 0.1mg/ml LDL and various concentrations of 7,8-NP, in Ham's F10 supplemented with 1.5µM $FeSO_4$. After nine hours, samples were analyzed for (a) protein hydroperoxides, (b) TBARS and (c) vitamin E. Significance is indicated from 0µM 7,8-NP. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

Additional experiments were conducted over 24 hours and indicated that 7,8-NP was able to extend the lag phase during THP-1 macrophage-mediated LDL oxidation in a concentration-dependent manner (Figure 5.11). This extension was observed whether monitoring protein hydroperoxide or TBARS formation and was most noticeable at lower 7,8-NP concentrations. A three hour lag phase, in the absence of 7,8-NP, doubled to six hours in the presence of 10μM 7,8-NP and then nine hours upon incubation with 20μM 7,8-NP. By contrast, the much larger concentration of 50μM 7,8-NP produced a lag phase that lasted only nine to twelve hours. Irrespective of the 7,8-NP incubation concentration or lag phase length, the subsequent propagation phase was associated with oxidation of both the lipid and protein components of LDL. This oxidation proceeded at rates similar to those observed in the absence of any 7,8-NP.

In agreement with the concentration-dependent extension of the protein hydroperoxide and TBARS lag phases, 7,8-NP was observed to delay the cell-mediated loss of LDL's vitamin E in a similar manner (Figure 5.11c). This effect was, once again, more prominent at the lower concentrations of 10μM and 20μM 7,8-NP. Even so, the 7,8-NP-mediated protection was so efficient at earlier time points that an almost complete retention of vitamin E was initially recorded for all 7,8-NP treatments. By the final stages, vitamin E oxidation occurred rapidly and at comparable rates, largely independent of the starting 7,8-NP concentration.

To complement the kinetic data obtained during these LDL oxidation studies, the oxidation of the exogenously added 7,8-NP to 7,8-dihydroxanthopterin (7,8-XP) was also monitored (Figure 5.12). 7,8-NP levels at the zero hour time point were always slightly lower than the stated preparation concentration. This is likely due to autoxidation during the preparation of the antioxidant solution and does not alter the general trends described below. As expected, neither 7,8-NP nor 7,8-XP were detectable in samples lacking exogenous 7,8-NP (data not shown) but the 10μM, 20μM and 50μM treatments all showed a time-dependent loss of 7,8-NP. In contrast to 7,8-NP, the concentration of 7,8-XP was difficult to determine. A 7,8-XP standard was obtained, but this compound was labile and had partially degraded during transport to New Zealand. Although this prevented 7,8-XP from being quantified to a micromolar value, an increase in the chromatographic area corresponding to 7,8-XP nevertheless confirmed that the depletion of 7,8-NP and the formation of 7,8-XP occurred concomitantly.

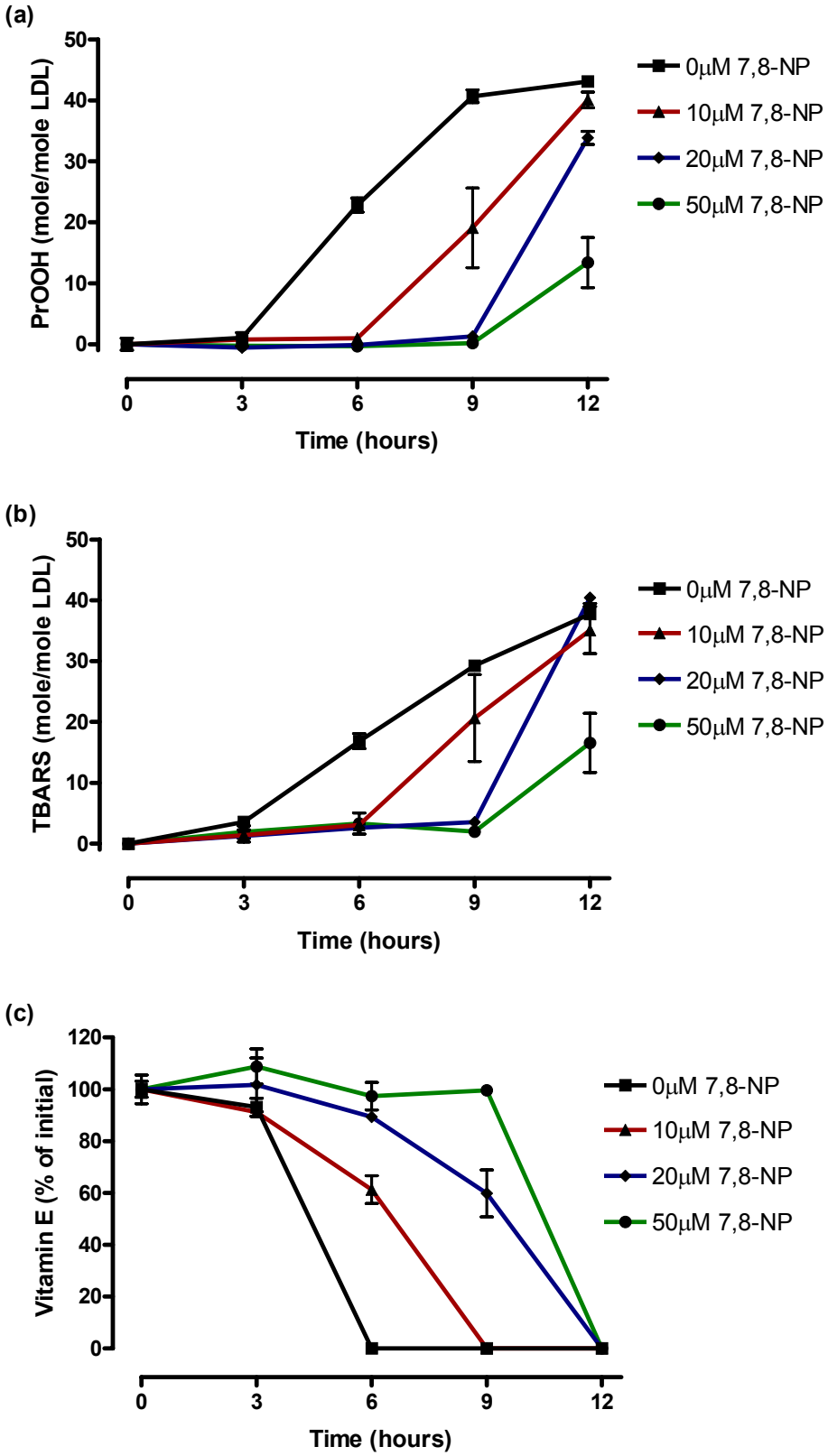


Figure 5.11 7,8-NP extends the lag phase during THP-1 macrophage-mediated LDL oxidation.

Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C, with 0.1mg/ml LDL and various concentrations of 7,8-NP, in Ham's F10 supplemented with 1.5μM $FeSO_4$. Samples were analyzed at various time points for an increase in (a) protein hydroperoxides and (b) TBARS and (c) a loss of vitamin E. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

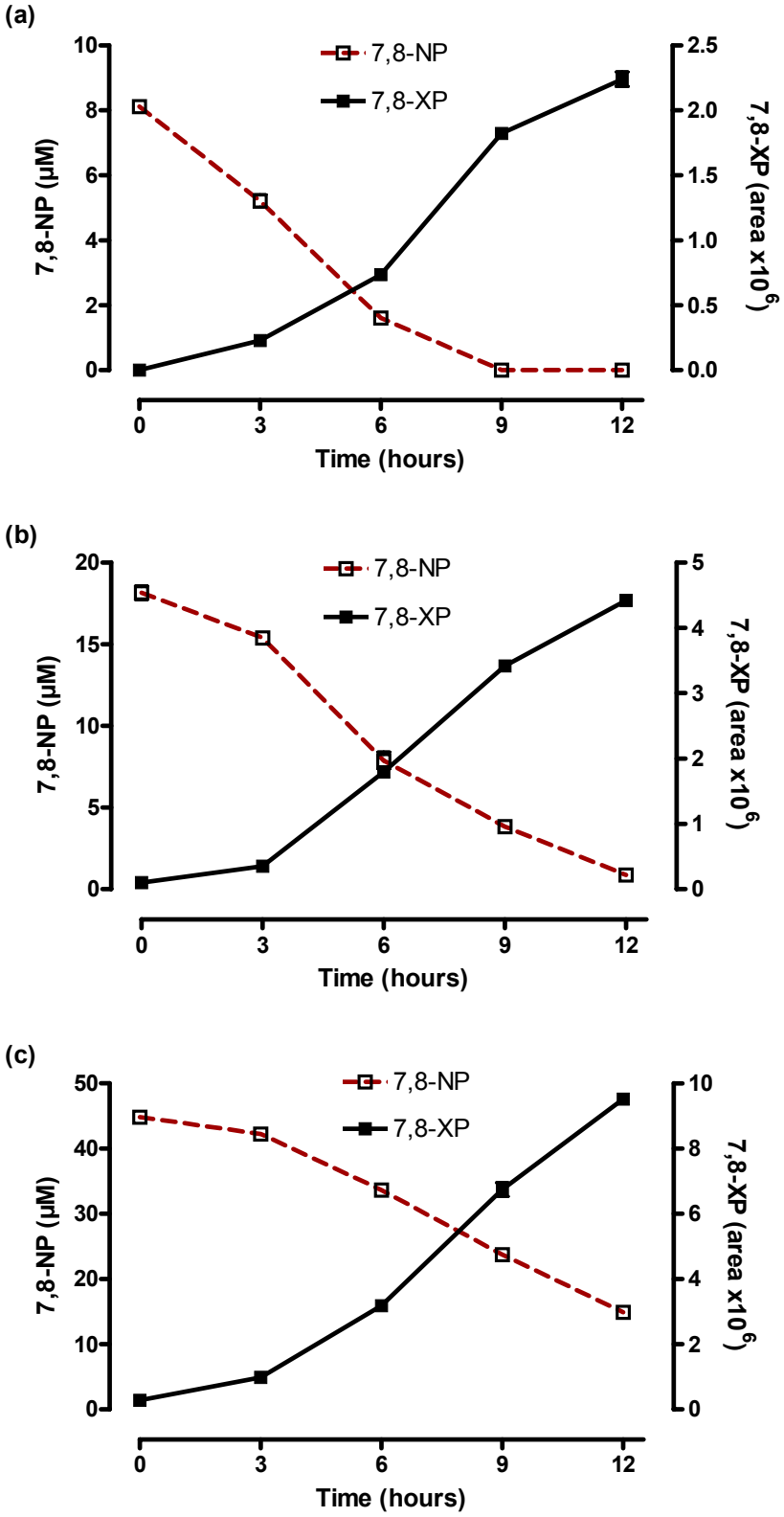


Figure 5.12 Oxidation of 7,8-NP during THP-1 macrophage-mediated LDL oxidation.

Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C in 1.5µM $FeSO_4$-supplemented Ham's F10, with 0.1mg/ml LDL and either (a) 10µM 7,8-NP, (b) 20µM 7,8-NP or (c) 50µM 7,8-NP. Samples were analyzed at various time points for the loss of 7,8-NP (displayed as a micromolar concentration) and the formation of 7,8-XP (displayed as area on the HPLC chromatograph). Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

Just as critically, 7,8-NP loss was also found to correlate with the cell-mediated oxidation of LDL. Figure 5.13 represents an incubation with 20μM 7,8-NP and provides an example of this relationship, with the almost complete consumption of 7,8-NP by nine hours corresponding to the end of the protein hydroperoxide and TBARS lag phase. A comparison of 7,8-NP and vitamin E oxidation indicated that loss of the former began almost immediately while loss of the latter was delayed until almost six hours. Both were ultimately 100% depleted at the same time point of 12 hours.

7,8-NP oxidation in iron(II)-supplemented Ham's F10 was further investigated by monitoring the impact of the presence and absence of both THP-1 macrophages and LDL on this process (Figure 5.14). In the absence of either of these components, a slight decrease in the level of 7,8-NP occurred over 12 hours. An identical trend was observed upon incubation of the 20μM 7,8-NP with THP-1 macrophages. In both cases, a small but corresponding increase in the amount of 7,8-XP was also noted. Co-incubation with LDL rather than cells produced more extensive 7,8-NP oxidation and 7,8-XP formation, with such reactions being most apparent between nine and twelve hours. An even more enhanced rate of 7,8-NP oxidation was observed upon addition of cells to the 7,8-NP/LDL incubation mixture. This oxidation was evident at all time points and corresponded to an increased rate of 7,8-XP formation.

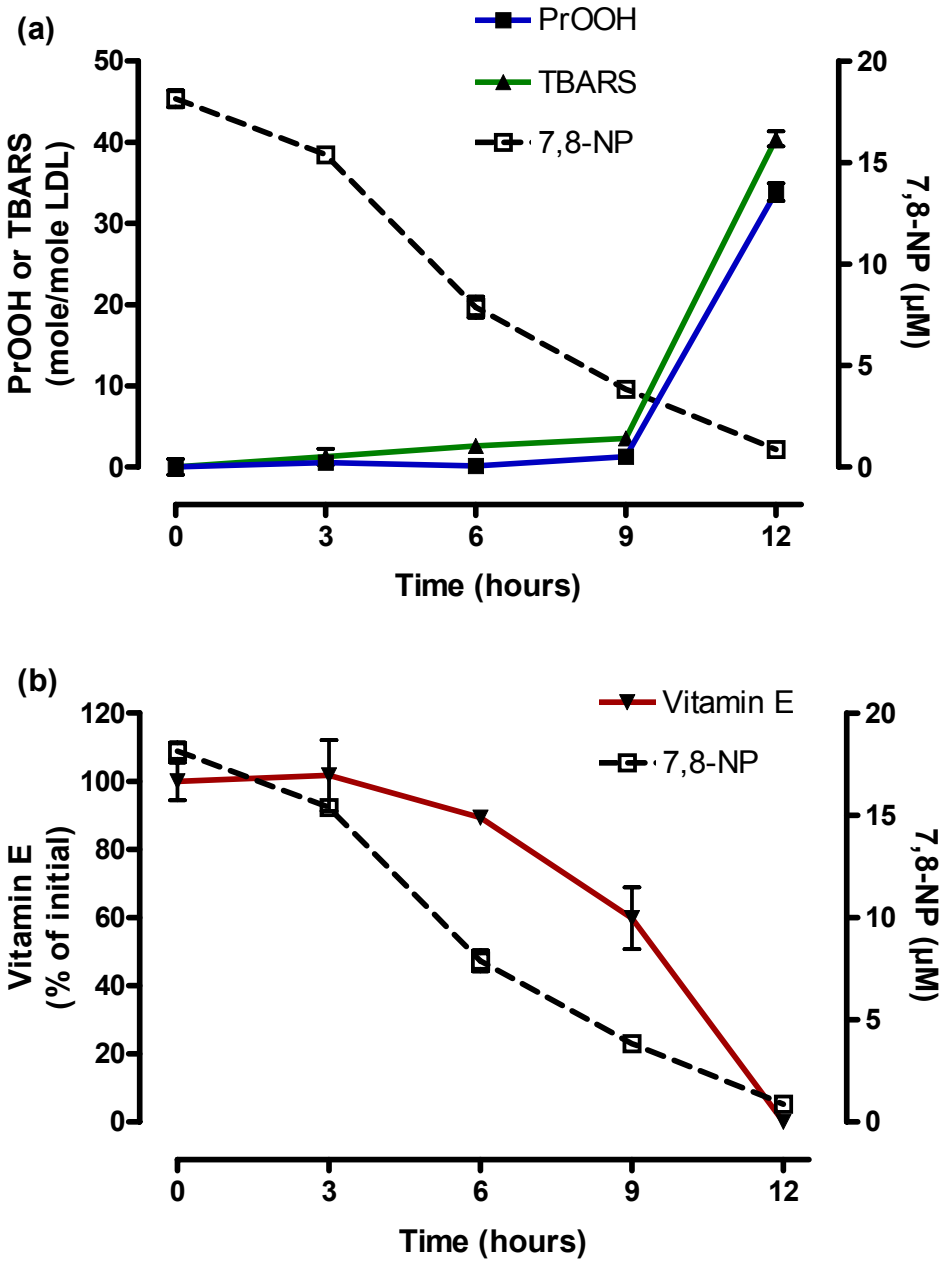


Figure 5.13 Correlation between the oxidation of 7,8-NP and THP-1 macrophage-mediated LDL oxidation.

Adherent THP-1 macrophage-like cells ($1x10^5$/ml) were incubated at 37°C, with 0.1mg/ml LDL and 20μM 7,8-NP, in Ham's F10 supplemented with 1.5μM $FeSO_4$. At various time points, 7,8-NP oxidation was compared to (a) the formation of protein hydroperoxides and TBARS and (b) the loss of vitamin E. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

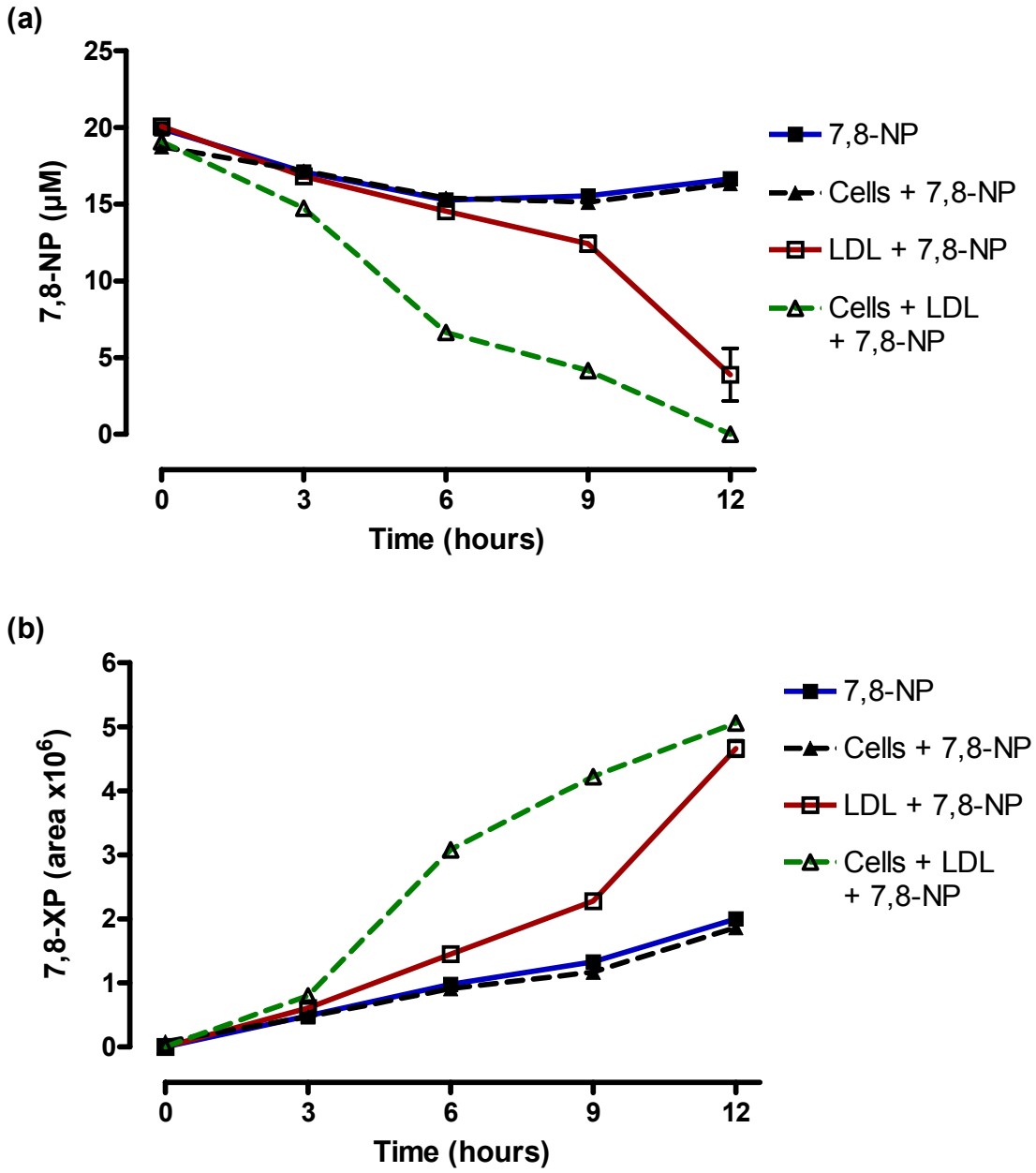


Figure 5.14 Effect of THP-1 cells and LDL on the oxidation of 7,8-NP.
20µM 7,8-NP was dissolved in Ham's F10 supplemented with 1.5µM $FeSO_4$. The 7,8-NP was incubated at 37°C, either alone or in combination with 0.1mg/ml LDL and/or $1x10^5$/ml THP-1 macrophage-like cells. Samples were analyzed at various time points for the (a) loss of 7,8-NP (displayed as a micromolar concentration) and (b) formation of 7,8-XP (displayed as area on the HPLC chromatograph). Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

5.2.4 7,8-NP, IFN-γ and TPMET

Consistent protection against cell-mediated LDL oxidation requires a pre-incubation of 7,8-NP with THP-1s not the lipoprotein (Gieseg & Cato, 2003). 7,8-NP-mediated protection of vitamin E also yielded different kinetics in the presence of THP-1 macrophages (Figure 5.11) compared to cell free conditions (Gieseg *et al.*, 1995). These observations raised questions regarding 7,8-NP's mechanism of inhibiting macrophage-mediated LDL oxidation and whether it involved some, as yet unidentified, direct interaction with the cells. The transplasma membrane electron transport (TPMET) system is suggested to play a critical role in atherosclerosis, transferring electrons from intracellular NADH to the extracellular transition metals that can subsequently promote the oxidation of LDL (Garner *et al.*, 1997b; Baoutina *et al.*, 2000; Baoutina *et al.*, 2001b). As such, it was deemed important to investigate whether any interaction between 7,8-NP and the TPMET system was responsible for this pteridine's effective inhibition of THP-1 macrophage-mediated LDL oxidation.

Two assays were used to monitor the extent of cellular reduction, with one based on the reduction of ferricyanide to ferrocyanide and the second based on the reduction of cupric ions to cuprous ions. The latter assay is susceptible to falsely low readings because, unlike ferricyanide, cupric ions are not impermeable to cells (Baoutina *et al.*, 2001b). Subjecting cupric ions to a control incubation in the presence and absence of THP-1 macrophages before initiating ascorbate-mediated reduction alleviated this concern by confirming a cuprous ion recovery of over 98% (data not shown).

Total cellular reducing activity was measured for both assays after a two hour incubation of cells in HBSS. This activity represents the combined reduction of both the TPMET system and any cellular reducing equivalents released into the medium. Assaying the reducing potential of cell-conditioned medium enables more direct measurement of the latter. Thus, TPMET activity can be indirectly determined by calculating the difference between the total cellular reduction and the activity in the conditioned medium. Initial studies simply monitored the total cellular reduction, confirming that both the ferricyanide and cupric ion reducing assays yielded similar trends (Figure 5.15). Reducing activity and cell concentration were shown to be positively associated, with $5x10^5$ cells/ml representing a suitable THP-1 macrophage concentration for subsequent experiments.

The extent of total cellular reduction was found to remain relatively constant, from one experiment to the next, using THP-1 macrophages at this concentration of $5x10^5$/ml.

Reducing activity directly attributed to the cell-conditioned medium was also generally consistent. Reduction in the cell-conditioned medium accounted for between 10-25% and 40-60% of the total cellular reduction that occurred, as measured by the ferricyanide and copper assays, respectively (for example, Figure 5.16 & Figure 5.17). Exceptions to this general trend were occasionally observed. In these instances, total cellular reduction levels were unchanged but reduction in the cell-conditioned medium varied anywhere between 0% and 85% of the total activity (data not shown).

Both thiol-containing compounds and superoxide have been suggested as likely reductants to be released into the medium (Garner *et al.*, 1997b; Baoutina *et al.*, 2001b). However, thiol levels in the supernatant remained constant between experiments while superoxide was not produced by THP-1 macrophages, as measured by both the cytochrome C reduction and nitroblue tetrazolium (NBT) assays (data not shown). This inability to identify the cause of the occasional variability in the activity of cell-secreted reductants was ultimately determined to have little consequence for subsequent experiments investigating the impact of 7,8-NP and IFN-γ on TPMET activity.

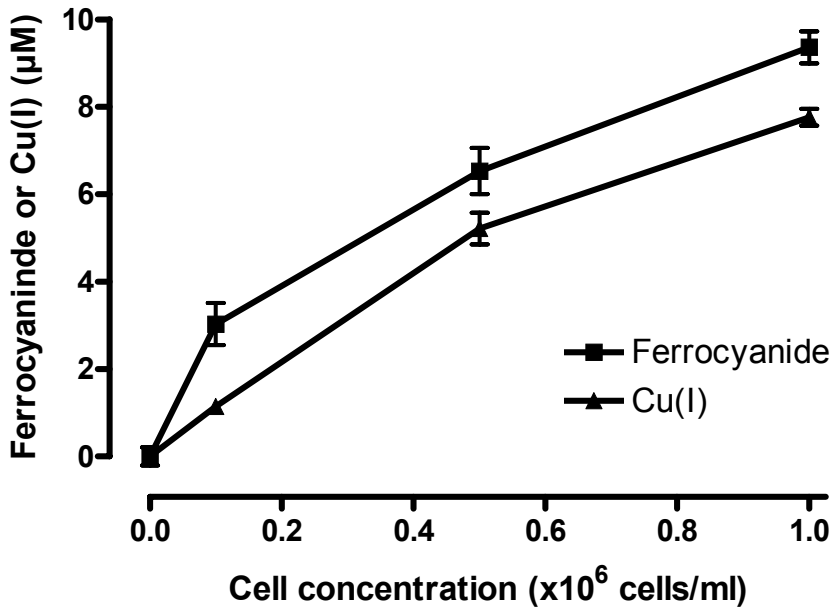


Figure 5.15 TPMET activity of THP-1 macrophage cells.
Adherent THP-1 macrophage-like cells were incubated at 37°C at various concentrations in HBSS for two hours in the presence of either ferricyanide or cupric chloride and BCS. Samples were then analyzed spectrophotometrically for reduction to ferrocyanide or cuprous ions, respectively, by the TPMET assays. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

Micromolar concentrations of 7,8-NP were found to strongly interfere with both reduction assays (data not shown) and this problem could only be resolved by the introduction of a 7,8-NP pre-incubation step. Cells were subsequently washed in PBS to remove all traces of 7,8-NP before initiating the two hour TPMET assay. Irrespective of the inclusion of the pre-incubating step with 50μM 7,8-NP, the level of reduction due to reductants in the conditioned medium or to TPMET remained unchanged (Figure 5.16). This lack of effect was observed whether pre-incubating for two or six hours and was associated with both the ferricyanide reduction and cupric ion reduction assays.

In contrast to 7,8-NP, neopterin's interference during the reduction assays was minimal (data not shown). This ensured that the low nanomolar quantities of 7,8-NP and neopterin detected during IFN-γ incubations would not strongly interfere with TPMET analysis. Following a pre-incubation with 500U/ml IFN-γ, THP-1 macrophages were therefore washed and incubated during the two hour ferricyanide and cupric ion reduction assays in the presence of fresh IFN-γ. Despite continuous exposure to IFN-γ, no significant difference was observed between incubations conducted in the presence and absence of this cytokine (Figure 5.17). This lack of effect was observed whether monitoring TPMET activity or the activity of reductants in the cell-conditioned medium. It was also independent of the assay system, with similar results obtained for both ferricyanide and cupric ion reduction.

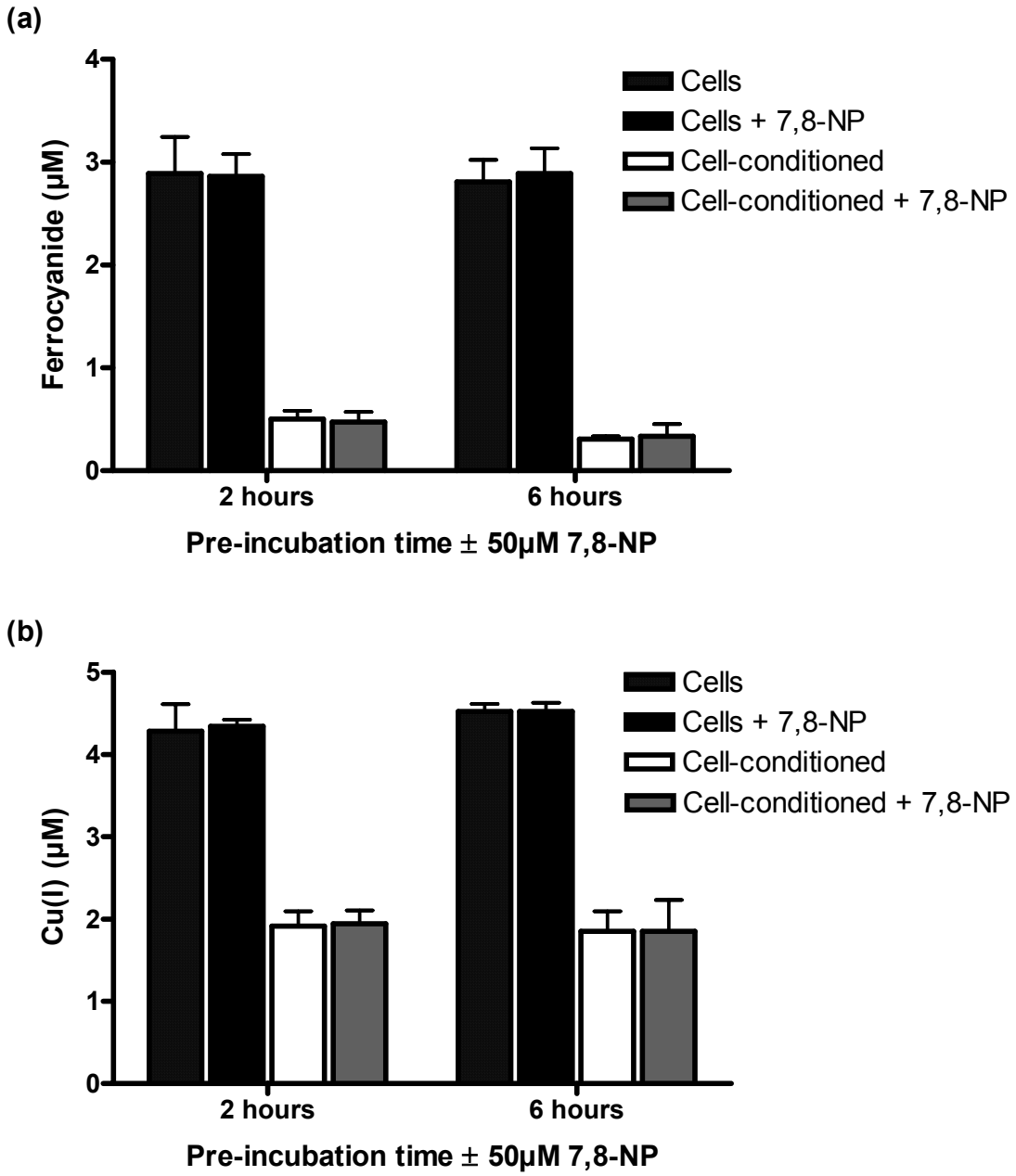


Figure 5.16 Effect of 7,8-NP on the TPMET activity of THP-1 macrophage cells.
Adherent THP-1 macrophage-like cells ($5x10^5$ cells/ml) were pre-incubated at 37°C in RPMI for either two or six hours with or without 50µM 7,8-NP. Wells were subsequently washed and incubated in HBSS for two hours in the presence of (a) ferricyanide or (b) cupric chloride and BCS. Samples were then analyzed spectrophotometrically for reduction to ferrocyanide or cuprous ions, respectively, by the TPMET assays. Cell-conditioned medium was similarly monitored for each assay. ANOVA analysis revealed no statistical significance between the presence and absence of 7,8-NP. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

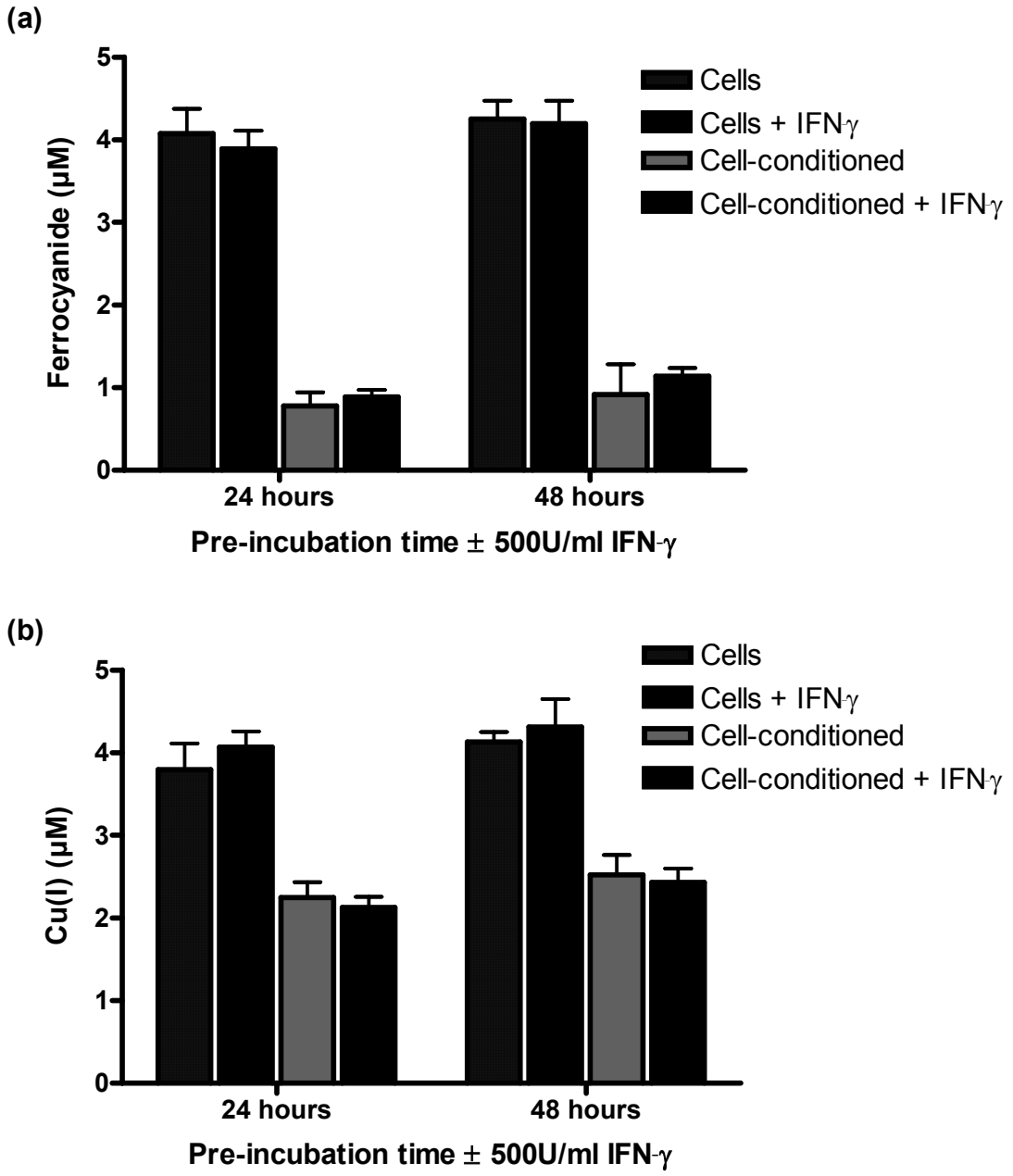


Figure 5.17 Effect of IFN-γ on the TPMET activity of THP-1 macrophage cells.
Adherent THP-1 macrophage-like cells ($5x10^5$ cells/ml) were pre-incubated at 37°C in RPMI for either 24 or 48 hours with or without 500U/ml IFN-γ. Wells were subsequently washed and incubated in HBSS for two hours in the presence of fresh IFN-γ and (a) ferricyanide or (b) cupric chloride and BCS. Samples were then analyzed spectrophotometrically for reduction to ferrocyanide or cuprous ions, respectively, by the TPMET assays. Cell-conditioned medium was similarly monitored for each assay. ANOVA analysis revealed no statistical significance between the presence and absence of IFN-γ. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

5.2.5 Effect of IFN-γ on THP-1 Macrophage-Mediated LDL Oxidation

In an attempt to identify optimal conditions for monitoring the effect of endogenous 7,8-NP on THP-1 macrophage-mediated LDL oxidation, cells were incubated in the presence of 500U/ml IFN-γ and monitored for total pterin (neopterin plus 7,8-NP) production at various times (Figure 5.18). Levels of total pterin reached significance in the supernatant after 36 hours while pterin in the cell extract became significant at 24 hours. This provides reasonable agreement with the approximately 30 hours required by GTP cyclohydrolase I for maximal activation (Wachter *et al.*, 1992). While a plateau in cell extract pterin concentrations was noted after 36 hours, supernatant levels continued to increase at least up to 48 hours. As a result, an incubation time of 48 hours was chosen as a suitable and convenient period of IFN-γ pre-incubation during subsequent experiments.

An additional control confirmed that LDL had no significant effect on pterin levels in either the supernatant or cell extract (Table 5.1).

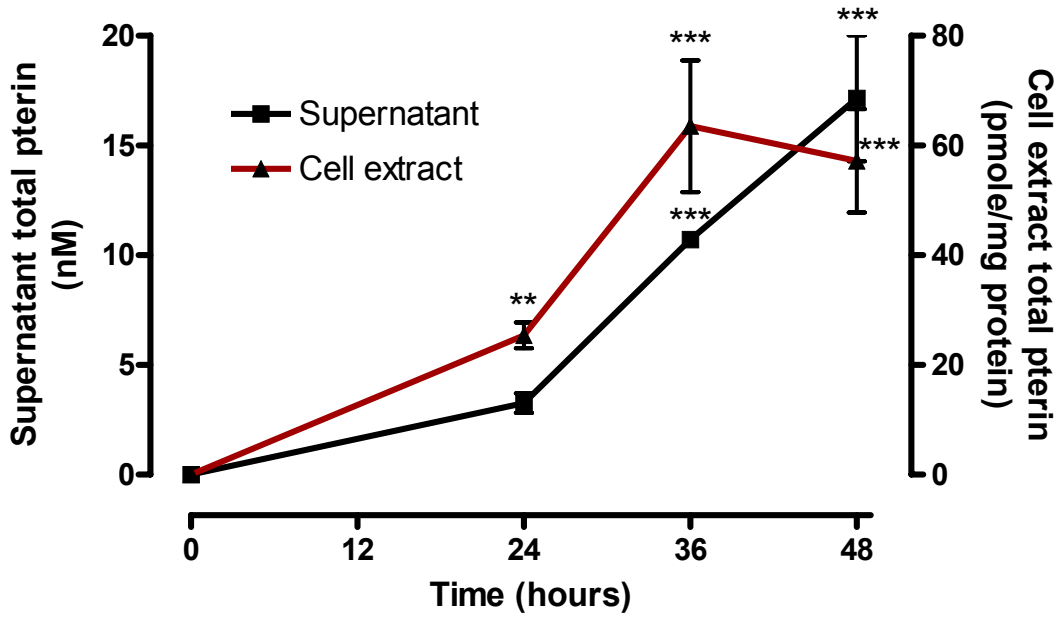


Figure 5.18 Time course of pterin production by THP-1 macrophages.
Adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml) were incubated at 37°C in RPMI with 500U/ml IFN-γ. At various time points, total pterin (neopterin plus 7,8-NP) levels were measured in the supernatant and cell lysate. Significance is indicated from time zero. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

Table 5.1 Effect of LDL oxidation on THP-1 macrophage pterin levels.
Adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml) were incubated at 37°C in RPMI with 500U/ml IFN-γ. After 48 hours, the cells were washed and LDL oxidation was initiated by the addition of Ham's F10 supplemented with 1.5μM $FeSO_4$, 0.1mg/ml LDL and fresh IFN-γ. Controls included LDL only and cells only, either in the presence or absence of IFN-γ. After 24 hours, total pterin (neopterin plus 7,8-NP) levels were measured in the supernatant and cell lysate of all samples. ANOVA analysis revealed no statistical significance between the presence and absence of LDL. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

	Cell extract (pmole/mg protein)	**Supernatant (nM)**
LDL + $FeSO_4$	N/A	0 ± 0
LDL + $FeSO_4$ + IFN-γ	N/A	0 ± 0
Cells	0 ± 0	0 ± 0
Cells + LDL + $FeSO_4$	0 ± 0	0 ± 0
Cells + IFN-γ	48.21 ± 2.32	6.54 ± 1.03
Cells + IFN-γ + LDL + $FeSO_4$	54.62 ± 7.05	5.27 ± 0.48

Initial experiments monitoring the role of IFN-γ on THP-1 macrophage-mediated LDL oxidation followed the protocol of Gieseg & Cato (2003). After a pre-incubation in the RPMI with 500U/ml IFN-γ, the stimulated THP-1 macrophages were washed and subjected to a nine hour incubation in Ham's F10 supplemented with ferrous ions, LDL and fresh IFN-γ. These conditions failed to prevent the formation of protein hydroperoxides and TBARS on LDL, irrespective of whether the THP-1 macrophages were pre-incubated with IFN-γ for 24 or 48 hours (Figure 5.19). Similarly, vitamin E was completely depleted after subjecting the LDL to cell-mediated oxidation and neither a 24 hour nor a 48 hour pre-incubation with IFN-γ prevented its loss (data not shown).

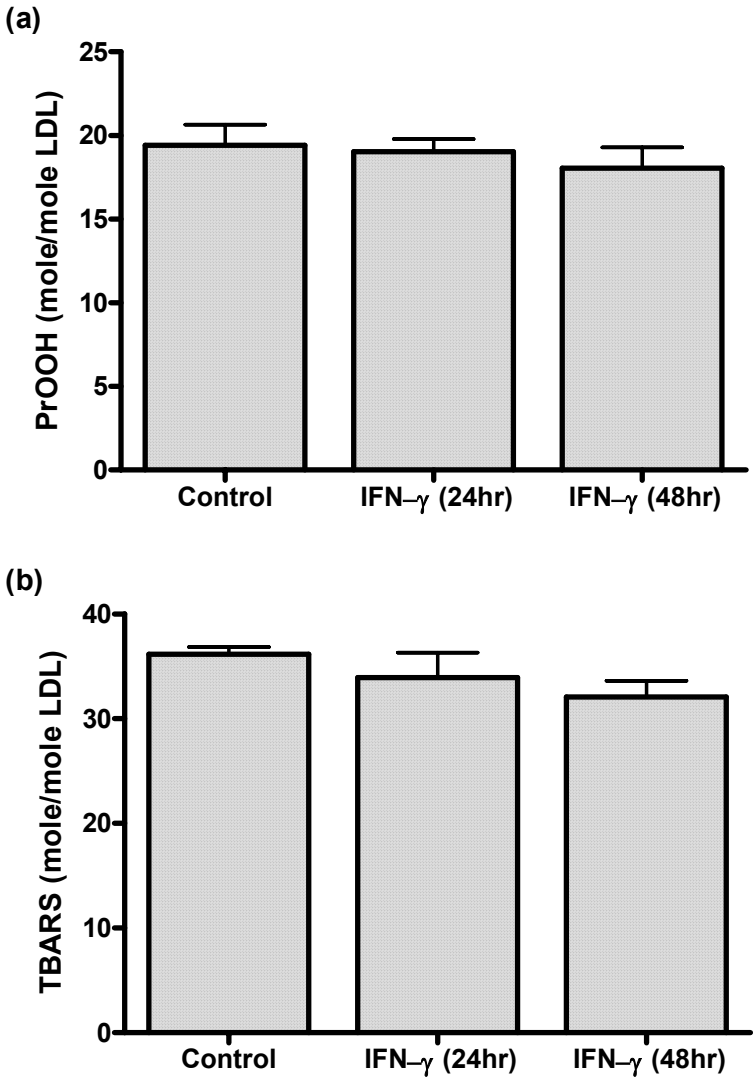


Figure 5.19 THP-1 macrophage-mediated LDL oxidation after a pre-incubation in RPMI with IFN-γ.

Adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml) were pre-incubated in RPMI for 24 or 48 hours in the presence or absence (control) of 500U/ml IFN-γ. The experiment was subsequently initiated by removal of RPMI and replacement with Ham's F10 supplemented with 1.5μM $FeSO_4$, 0.1mg/ml LDL and fresh IFN-γ. Samples were analyzed after nine hours for an increase in (a) protein hydroperoxides and (b) TBARS. ANOVA analysis revealed no statistical significance from the control. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of two separate experiments.

It remained possible that the change of media between the pre-incubation and incubation steps obscured any potential protective effect of IFN-γ. The chances of observing inhibition were also likely to be lessened at faster rates of cell-mediated LDL oxidation. Provided the THP-1 macrophages survived for extended periods in Ham's F10, these issues could be resolved by continuous exposure to Ham's F10 during both the pre-incubation and incubation steps. Viability in the presence and absence of IFN-γ was confirmed during 48 and 72 hour incubations of THP-1 macrophages in Ham's F10, with the latter time point representing the combined pre-incubation and incubation period. A comparison against the control incubation in RPMI indicated that a small drop in viability did occur over time (Figure 5.20). After 72 hours in Ham's F10, this had approached 86.8 ± 1.7% and 90.9 ± 0.5% of control values according to the MTT and trypan blue exclusion assays, respectively. Viability loss was slightly reduced in the presence of IFN-γ, but this effect failed to reach significance for either viability assay. Thus, THP-1 macrophages retained a reasonable level of viability during prolonged incubations in Ham's F10, and the addition of IFN-γ had little impact on this cell survival.

These cells were then investigated once more for their ability to inhibit LDL oxidation when stimulated with 500U/ml IFN-γ. This time the entire experiment was conducted in Ham's F10, incorporating a 48 hour pre-incubation followed by a 36 hour incubation in the presence of transition metals and LDL. As expected, the rate of THP-1 macrophage-mediated LDL oxidation was slowed by the inclusion of a 48 hour pre-incubation in Ham's F10 (Figure 5.21). The lag phase was extended from three to six hours and LDL peroxidation products did not reach maximal levels until 18-24 hours. Stimulating THP-1 macrophages with IFN-γ appeared to marginally slow protein hydroperoxide and TBARS formation on LDL, but this never approached significance. Furthermore, the effect was not consistently observed, with some repeats of the experiment showing no detectable difference between treatments (data not shown). Likewise, the rate of vitamin E oxidation was unchanged when incubating THP-1 macrophages with IFN-γ compared to the unstimulated control.

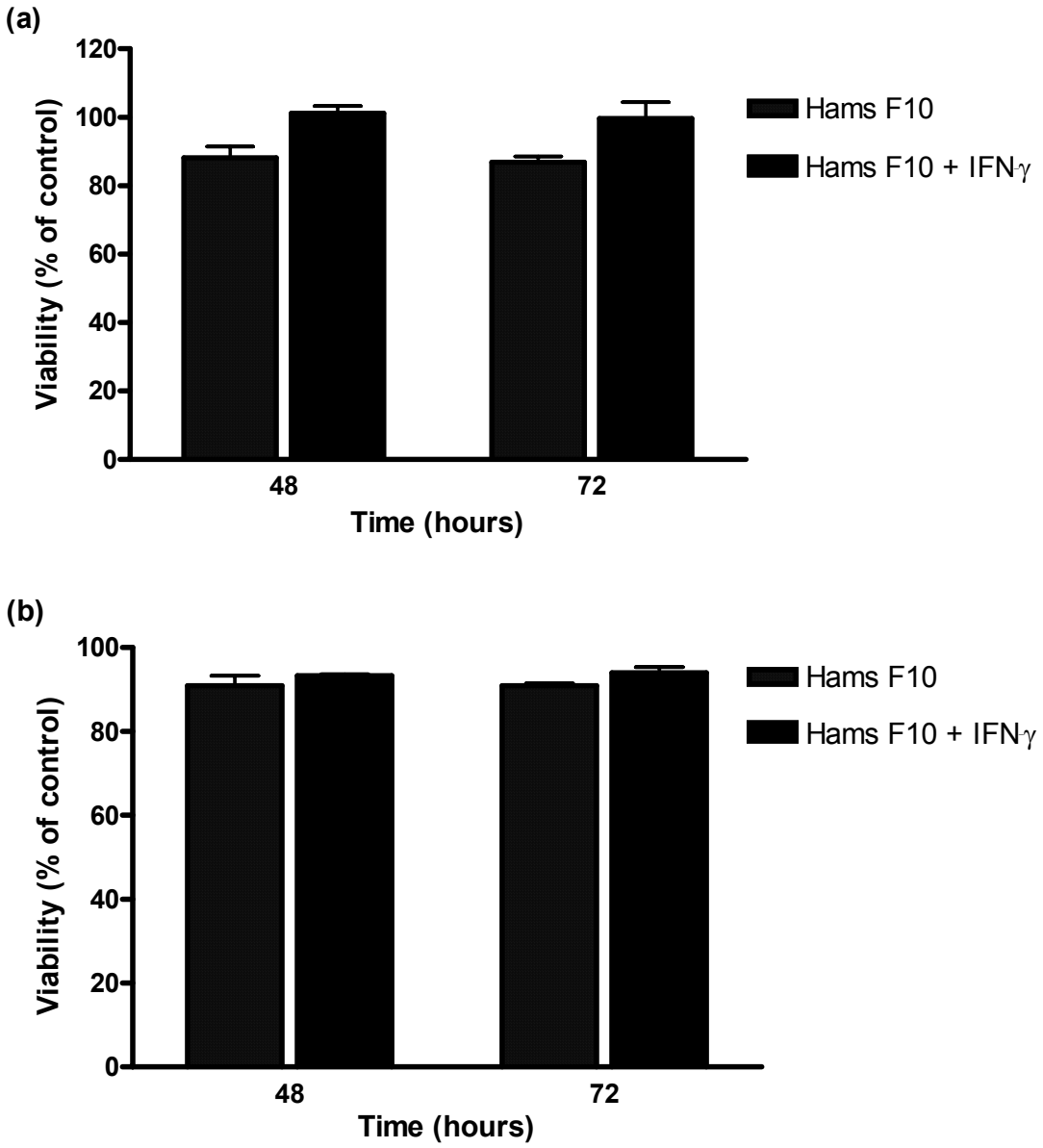


Figure 5.20 THP-1 macrophage viability in Ham's F10 medium.
Adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml) were incubated in Ham's F10, supplemented with 1.5μM $FeSO_4$, in the presence or absence of 500U/ml IFN-γ. Controls incubated in RPMI for the entire experiment were also prepared. After 48 and 72 hours, cell viability was analyzed via (a) MTT and (b) trypan blue exclusion staining. ANOVA analysis revealed no statistical significance between treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

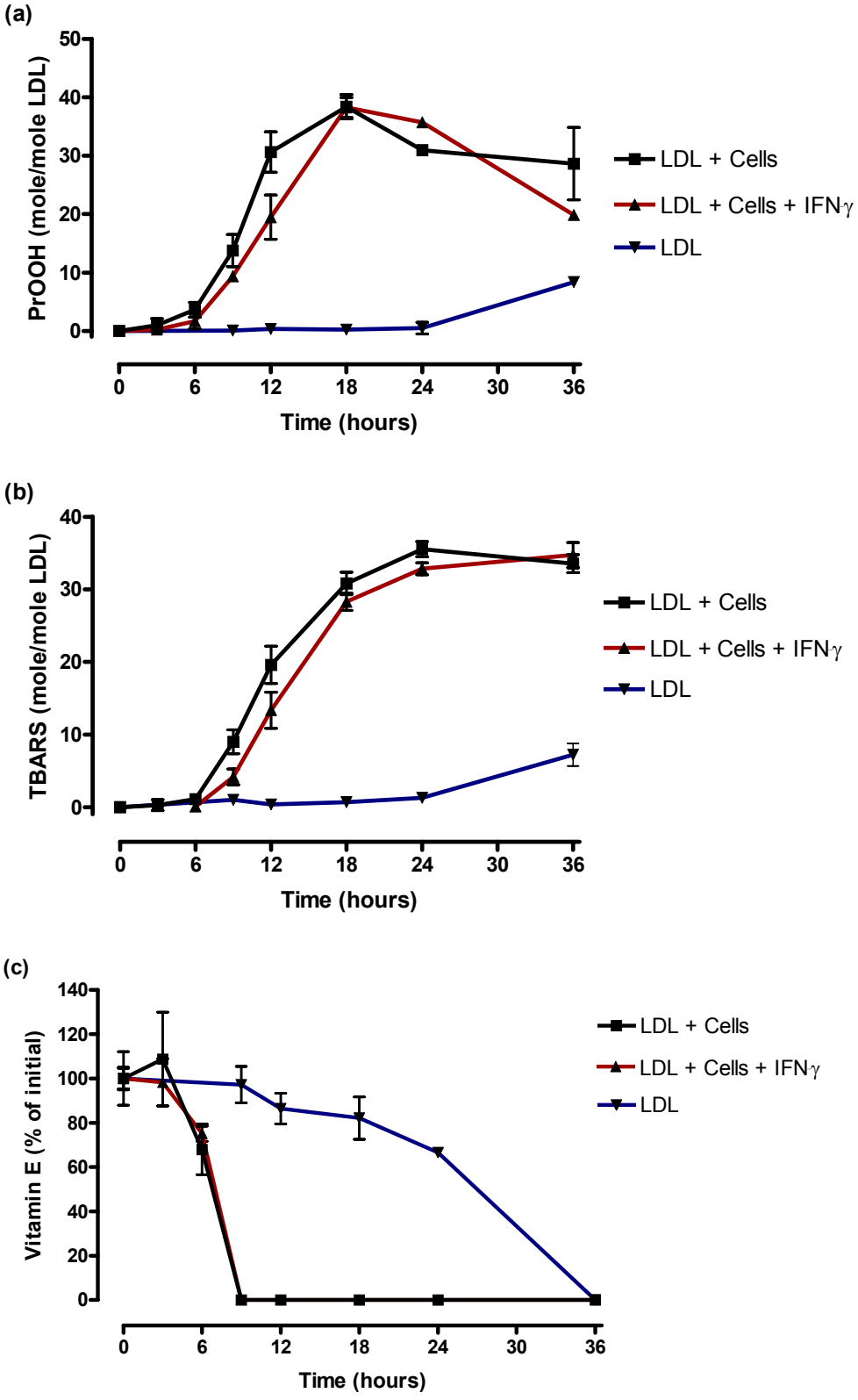


Figure 5.21 IFN-γ does not inhibit THP-1 macrophage-mediated LDL oxidation. Adherent THP-1 macrophage-like cells ($1x10^5$ cells/ml) were pre-incubated in chelexed Ham's F10 for 48 hours in the presence or absence of 500U/ml IFN-γ. The experiment was subsequently initiated by the addition of 4.5μM $FeSO_4$, 0.01μM $CuCl_2$ and 0.1mg/ml LDL. An LDL only treatment was also included. Samples were analyzed at various time points for an increase in (a) protein hydroperoxides, (b) TBARS and (c) the loss of vitamin E. ANOVA analysis revealed no statistical significance between cell treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

5.2.6 Protein Hydroperoxide Formation on LDL During Oxidation Mediated by HMDMs

While THP-1 macrophages had clearly been shown to promote the formation of protein hydroperoxides on LDL, it remained to be confirmed whether this was specific to the THP-1 cell line or whether it was a general feature of macrophage-mediated LDL oxidation. To answer this question, the more physiological HMDMs were incubated in the presence of transition metals and LDL. HMDMs were initially plated at four different densities in 12 well plates and allowed to mature over 14 days. TBARS formation during HMDM-mediated LDL oxidation was subsequently monitored in 3ml of Ham's F10 to provide an indication of the most suitable cell concentration for additional experiments (Figure 5.22). All cell concentrations were found to promote LDL oxidation but $1x10^6$ cells/ml ($3x10^6$ cells/well) was ultimately chosen for future studies. This concentration promoted a rate of oxidation that provided adequate time for sampling and for the detection of all critical kinetic phases in the process of LDL modification. Just as critically, $3x10^6$ cells/well was the lowest density regularly observed to yield a healthy monolayer of cells in the 12 well Falcon plates (data not shown).

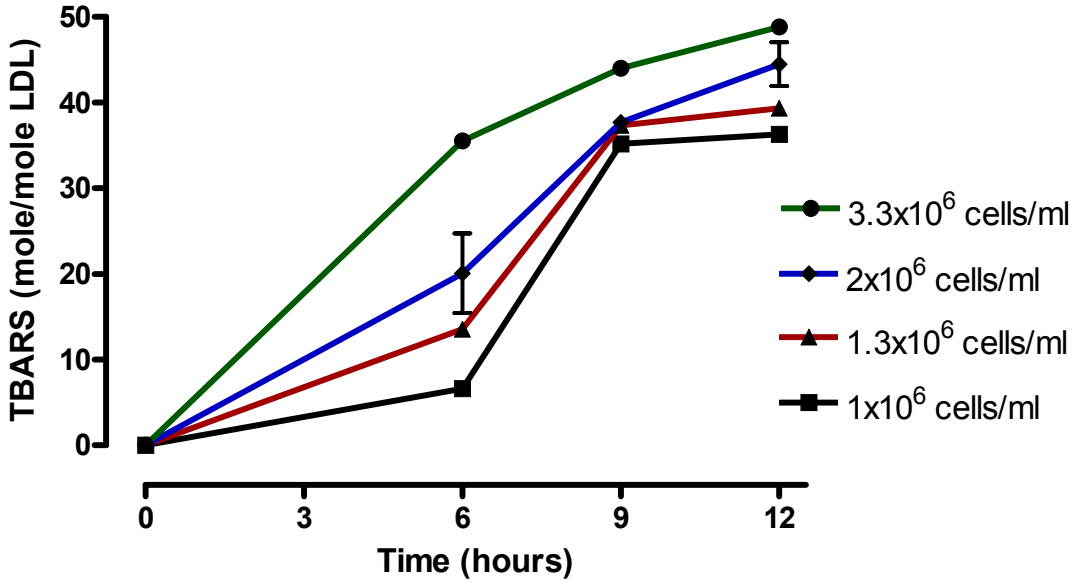


Figure 5.22 Effect of increasing cell concentrations on HMDM-mediated LDL oxidation

Increasing concentrations of HMDMs were incubated at 37°C with 0.1mg/ml LDL in 3ml of chelexed Ham's F10. The medium was supplemented with 4.5µM $FeSO_4$ and 0.01µM $CuCl_2$, and samples were analyzed at various time points for an increase in TBARS. Results are displayed as mean ± SEM of triplicate samples.

When LDL was exposed to this concentration of HMDMs, protein hydroperoxides were clearly detectable, reaching a maximum level of 30.8 ± 1.2 moles/mole LDL after 24 hours (Figure 5.23). As with THP-1 macrophage-mediated LDL oxidation, HMDMs promoted protein hydroperoxide formation in a manner that paralleled the generation of TBARS. Both parameters possessed a 12 hour lag phase and this corresponded to the complete loss of vitamin E between 12 and 18 hours. In agreement with the THP-1 macrophages, HMDMs were also confirmed to promote a significantly faster rate of protein hydroperoxide formation on LDL than the corresponding cell free treatments.

As expected, protein hydroperoxides, TBARS and vitamin E levels in the supernatant removed from cell only controls remained unchanged throughout the experiment.

Contrasting with the relative consistency of THP-1 macrophage-mediated LDL oxidation, variable rates of HMDM-mediated oxidation were commonly noted between batches of HMDMs. Thus, lag phases in the presence of $1x10^6$ HMDMs/ml were found to vary anywhere between three and 12 hours from one macrophage preparation to the next. Figure 5.22 and Figure 5.23 provide an example of this, with lag phases of approximately six hours and 12 hours, respectively, despite otherwise identical incubation conditions.

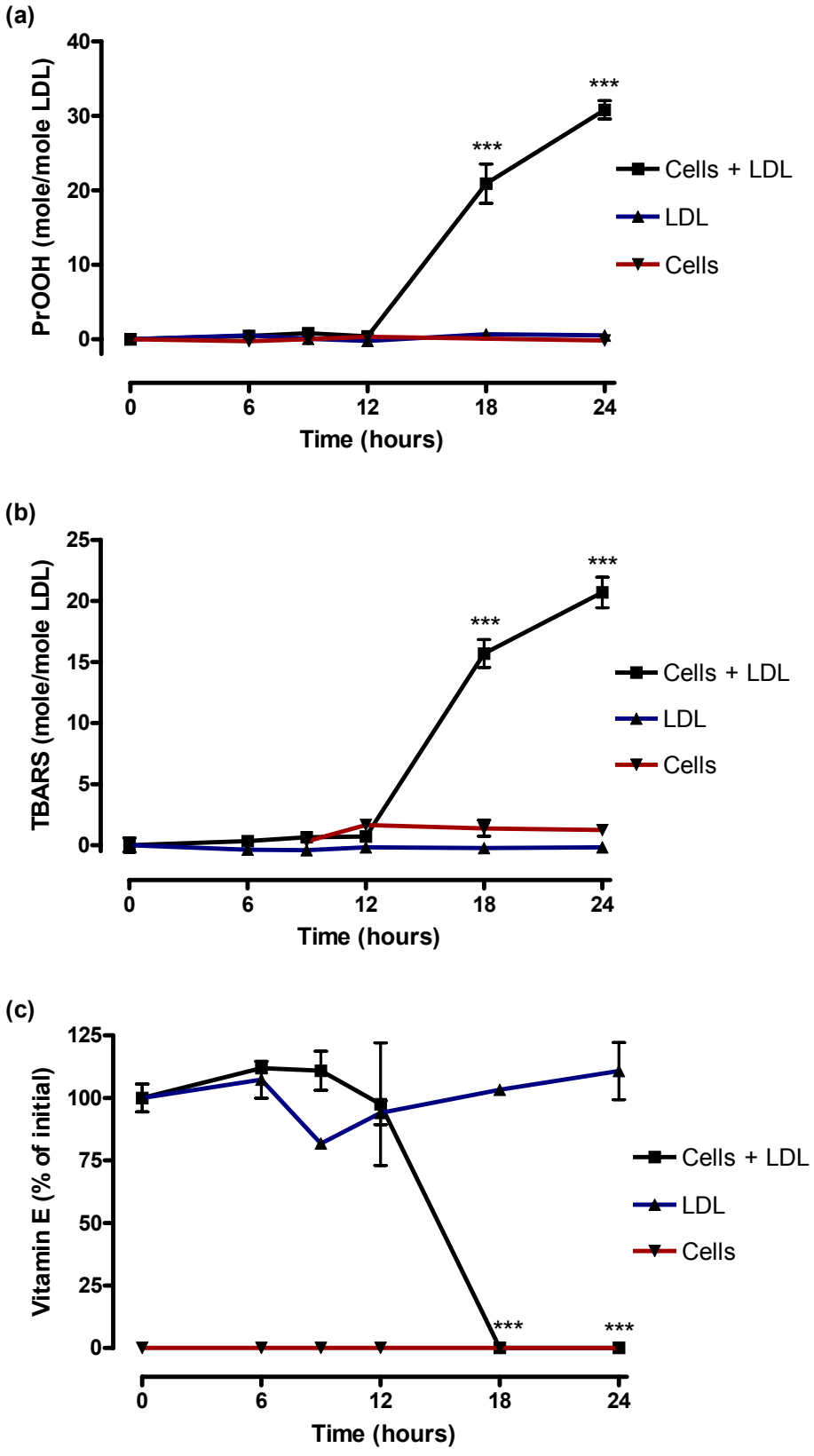


Figure 5.23 HMDM-mediated LDL oxidation.
HMDMs ($1x10^6$/ml) were incubated at 37°C with 0.1mg/ml LDL in Ham's F10 supplemented with 4.5μM $FeSO_4$ and 0.01μM $CuCl_2$. Controls included cells only and LDL only. Samples were analyzed at various time points for an increase in (a) protein hydroperoxides, (b) TBARS and (c) the loss of vitamin E. Significance is indicated from time zero. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

5.2.7 7,8-NP Inhibits Protein Hydroperoxide Formation During LDL Oxidation Mediated by HMDMs

The effect of 7,8-NP on cell-mediated LDL oxidation was studied using a similar protocol for both THP-1 macrophages and HMDMs. A five minute pre-incubation of HMDMs in chelexed Ham's F10, freshly supplemented with 4.5μM ferrous ions and 0.01μM cupric ions, was therefore followed by a further five minute pre-incubation with various concentrations of 7,8-NP. Samples were then incubated in the presence of 0.1mg/ml LDL for a period of time that corresponded to a peak in TBARS and protein hydroperoxide levels in the 0μM 7,8-NP control treatment. Due to the variable rates of HMDM-mediated LDL oxidation obtained between blood donors, a time course was conducted prior to each one of these 7,8-NP experiments to enable proper identification of this time point. An 18 hour incubation was required to reach this peak when using the HMDM preparation in Figure 5.24 (data not shown). Under these incubation conditions, protection against HMDM-mediated protein hydroperoxide and TBARS formation on LDL only became significant between 85-95μM 7,8-NP (Figure 5.24). In support of the almost complete retention of vitamin E at 100μM 7,8-NP, lipid and protein oxidation was also almost completely inhibited at this pteridine concentration.

While 100μM 7,8-NP provided strong inhibition in the current study, LDL incubated with other HMDM preparations appeared to require anywhere between 100-200μM 7,8-NP for a similar level of protection. Thus, the variability between HMDM donors is associated not only with differing rates of cell-mediated LDL oxidation but also with the concentration of 7,8-NP required for complete prevention of oxidation. However, no correlation between these two factors could be identified. For instance, HMDMs from two separate donors promoted a peak in LDL oxidation products after nine hours and 18 hours, but 100μM 7,8-NP was similarly effective in preventing the LDL oxidation induced by both these cells (data not shown).

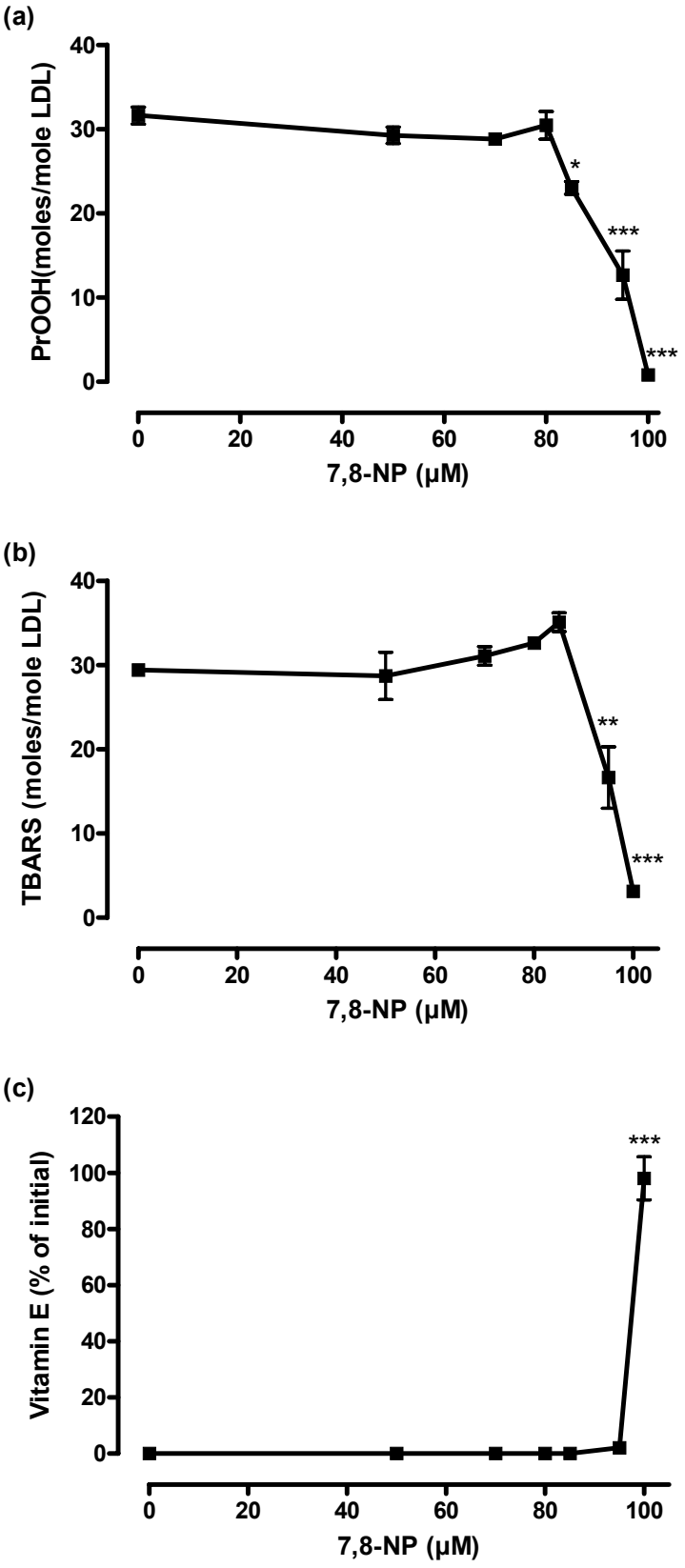


Figure 5.24 7,8-NP inhibits HMDM-mediated LDL oxidation.
HMDMs ($1x10^6$/ml) were incubated at 37°C, with 0.1mg/ml LDL and various concentrations of 7,8-NP, in chelexed Ham's F10 supplemented with 4.5µM $FeSO_4$ and 0.01µM $CuCl_2$. After 18 hours, samples were analyzed for (a) protein hydroperoxides, (b) TBARS and (c) vitamin E. Significance is indicated from the 0µM 7,8-NP treatment. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

5.2.8 IFN-γ Inhibits HMDM-Mediated LDL Oxidation

Werner-Felmayer *et al.* (1990) have previously noted that the THP-1 cell line and HMDMs display a similar pattern of pterin production, and this observation has been further confirmed in the current study (Figure 5.18 & Figure 5.25). Total HMDM pterin levels did not reach significance in both the supernatant and cell extract until 36 hours and, in the presence of $1x10^6$ cells/ml, pterin subsequently plateaued at approximately 17-20nM and 50-55pmole/mg protein in the supernatant and cell extract, respectively (Figure 5.25).

In further similarity to the THP-1 results, HMDM pterin production was not significantly affected by co-incubation with LDL or transition metals (Table 5.2).

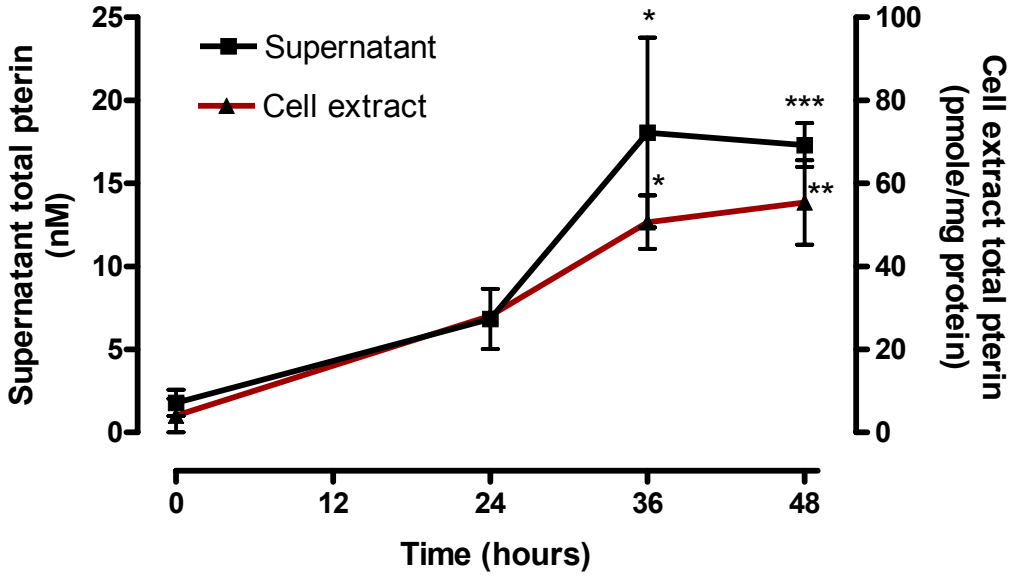


Figure 5.25 Time course of pterin production by HMDMs.
HMDMs ($1x10^6$ cells/ml) were incubated at 37°C in RPMI with 500U/ml IFN-γ. At various time points, total pterin (neopterin plus 7,8-NP) levels were measured in the supernatant and cell lysate. Significance is indicated from time zero. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

Table 5.2 Effect of LDL oxidation on HMDM pterin levels.
HMDMs ($1x10^6$/ml) were incubated at 37°C in chelexed Ham's F10 in the presence or absence of 500U/ml IFN-γ. After 48 hours, 4.5μM $FeSO_4$, 0.01μM $CuCl_2$ and 0.1mg/ml LDL were added to half the samples. Neopterin levels were subsequently measured in the supernatant and cell lysate of all samples after a further 24 hours. Significance is indicated between LDL oxidation samples and the corresponding LDL- and transition metal-free treatments. ANOVA analysis revealed no statistical significance between LDL oxidation samples and the corresponding LDL- and transition metal-free treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

	Cell extract (pmole/mg protein)	**Supernatant (nM)**
Cells	0 ± 0	2.75 ± 0.26
Cells + LDL + Metals	0 ± 0	0 ± 0
Cells + IFN-γ	48.95 ± 3.59	23.18 ± 1.14
Cells + IFN-γ + LDL + Metals	41.12 ± 5.43	23.40 ± 1.97

Like THP-1 macrophages, HMDMs were also able to survive for extended periods in Ham's F10 (Figure 5.26). As determined by trypan blue exclusion staining, viability in this medium remained at almost 100% compared to control cells incubated for up to 78 hours in the more nutrient rich RPMI. This lack of cell death was also observed in the presence of IFN-γ. Although the MTT assay did show a reduction in viability to 72.7 ± 1.7% after 78 hours in Ham's F10, the difference between cells incubated in the presence and absence of IFN-γ remained insignificant.

Given the viability of HMDMs in Ham's F10 over prolonged periods and the detection of neopterin under these conditions, the effect of IFN-γ on HMDM-mediated LDL oxidation could consequently be studied. Cells were stimulated with IFN-γ during a 48 hour pre-incubation in chelexed Ham's F10 before additional exposure to LDL and transition metals. In the absence of IFN-γ, HMDM-mediated LDL oxidation was associated with a lag phase of eight hours (Figure 5.27). The addition of IFN-γ extended this lag for both protein hydroperoxide and TBARS formation to 18-24 hours, with vitamin E being completely consumed between 24 and 36 hours. This contrasts with the total loss of vitamin E by 18 hours during LDL oxidation mediated by unstimulated HMDMs. The presence of IFN-γ actually retarded LDL oxidation to such an extent that the rate was even slower than that observed during cell free LDL oxidation.

A direct effect of IFN-γ on the oxidative process was eliminated by confirming that the presence of this cytokine had no effect on the rate of LDL oxidation under cell free conditions (data not shown).

The extent of inhibition mediated by IFN-γ-activated HMDMs during LDL oxidation showed some variability between donors. In general, this appeared to be independent of any changes in the rate of LDL oxidation mediated by unstimulated HMDMs. Although unstimulated HMDMs from two separate donors were both associated with a nine hour lag phase, the corresponding IFN-γ-stimulated HMDM treatments extended that lag phase to 18-24 hours in one experiment (Figure 5.27) and over 36 hours in the second experiment (data not shown).

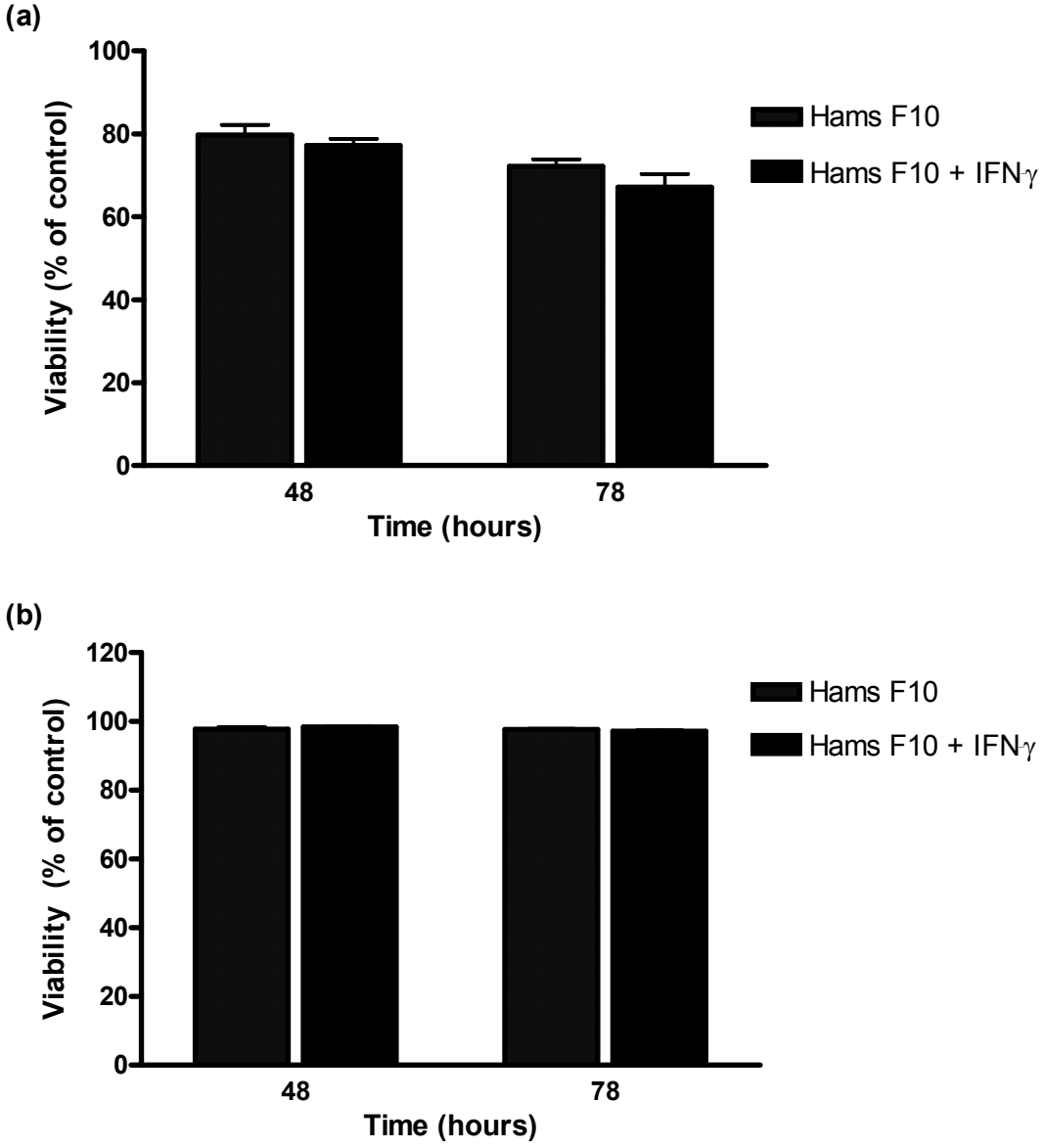


Figure 5.26 HMDM viability in Ham's F10 medium.
HMDMs ($1x10^6$ cells/ml) were incubated in Ham's F10, supplemented with 4.5μM $FeSO_4$ and 0.01μM $CuCl_2$, in the presence or absence of 500U/ml IFN-γ. Controls incubated in RPMI for the entire experiment were also prepared. After 48 and 78 hours, cell viability was analyzed via (a) MTT and (b) trypan blue exclusion staining. ANOVA analysis revealed no statistical significance between treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

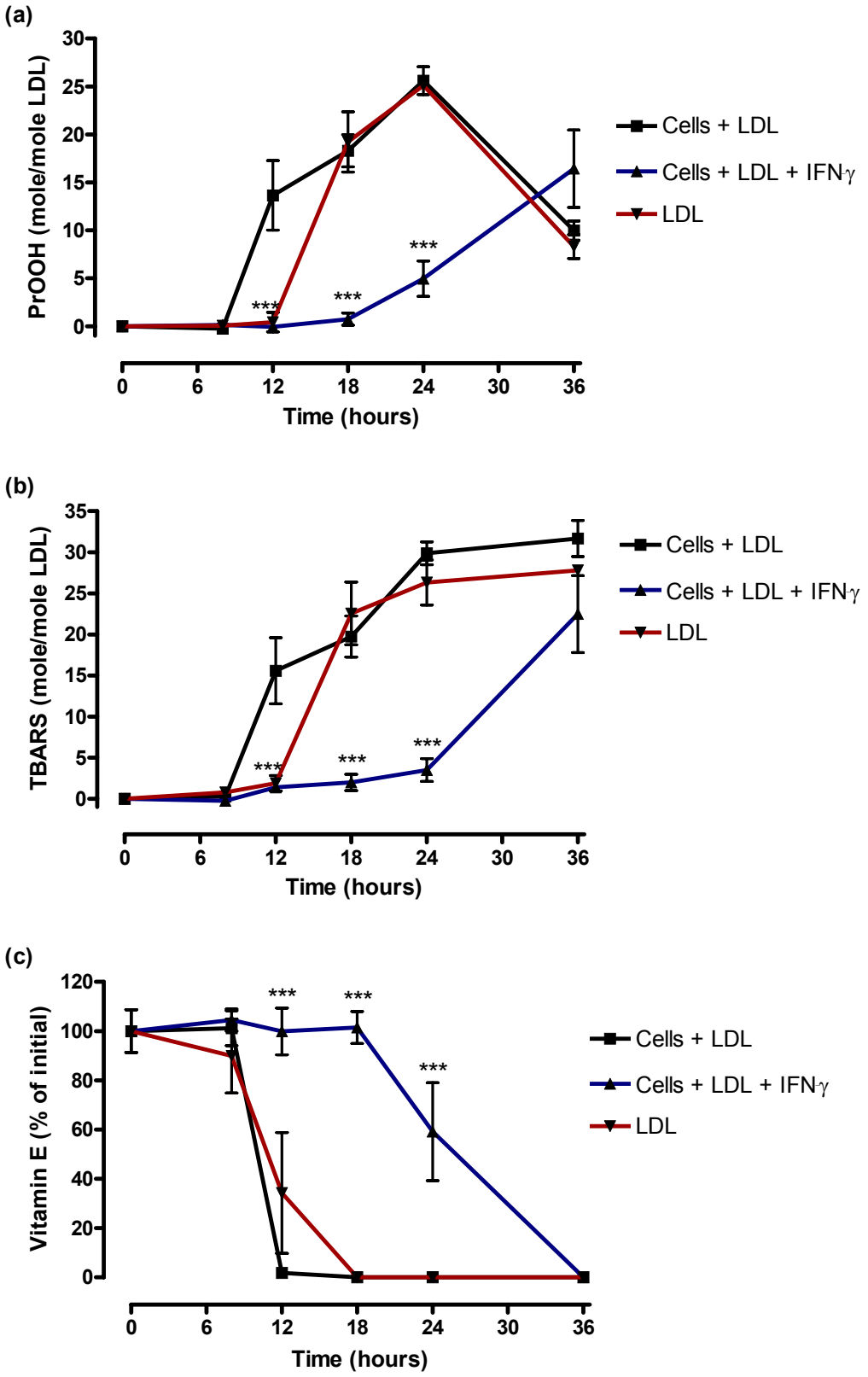


Figure 5.27 IFN-γ inhibits HMDM-mediated LDL oxidation.
HMDMs ($1x10^6$ cells/ml) were pre-incubated in chelexed Ham's F10 for 48 hours in the presence or absence of 500U/ml IFN-γ. The experiment was subsequently initiated by the addition of 4.5μM $FeSO_4$, 0.01μM $CuCl_2$ and 0.1mg/ml LDL. An LDL only treatment was also included. Samples were analyzed at various time points for an increase in (a) protein hydroperoxides, (b) TBARS and (c) a loss of vitamin E. Significance is indicated between cell treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

Irrespective of inter-experimental variability, the discovery of such a significant IFN-γ-mediated impact raised questions about the source of this inhibition and whether it required the continual presence of HMDMs or was caused by a cellular secretion. To test this, cell-conditioned Ham's F10 medium was prepared by incubating the HMDMs in the presence or absence of IFN-γ for 48 hours. LDL and 3μM cupric ions were then exposed to this conditioned medium for an additional incubation in the absence of cells (Figure 5.28). LDL oxidation in the unstimulated HMDM-conditioned medium exhibited kinetics identical to those observed during the corresponding cell free LDL oxidation. Both these treatments were associated with a lag phase of 12 hours, a similar rate of propagation and a decomposition phase that began after approximately 24 hours. This indicates that cell-mediated oxidation only occurs upon the continual exposure of LDL to macrophages. A completely different LDL oxidation trend was observed upon exposure of LDL to the conditioned medium derived from IFN-γ-stimulated HMDMs. Under these conditions, the TBARS lag phase was extended to 36 hours and the peak in LDL oxidation levels had not quite been attained by 48 hours.

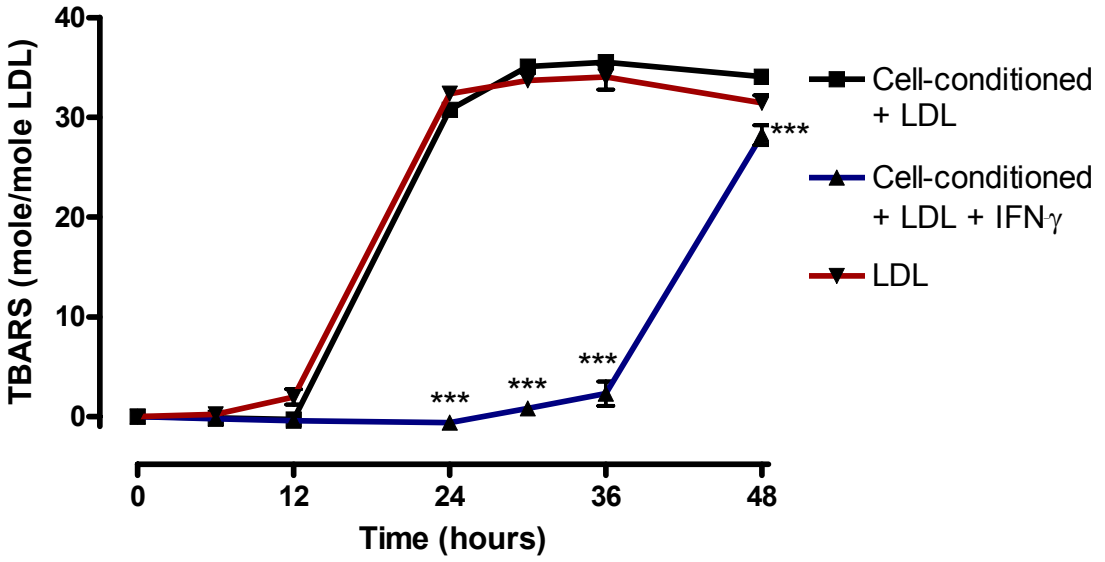


Figure 5.28 Conditioned medium removed from IFN-γ-treated HMDMs inhibits LDL oxidation.
HMDMs ($1x10^6$ cells/ml) were pre-incubated in chelexed Ham's F10 for 48 hours in the presence or absence of 500U/ml IFN-γ. The experiment was subsequently initiated by removing media from half the cells and incubating this cell-conditioned medium with 3μM $CuCl_2$ and 0.1mg/ml LDL. The remaining medium was kept with the HMDMs but treated similarly. An LDL only treatment was also included. Samples were analyzed at various time points for an increase in TBARS. Significance is indicated between cell-conditioned treatments. Results are displayed as mean ± SEM of triplicates from a single experiment, representative of three separate experiments.

5.3 Discussion

5.3.1 Transition Metals in LDL Oxidation

Transition metals have previously been shown to play a critical role in macrophage-mediated LDL oxidation (Leake & Rankin, 1990; Kritharides *et al.*, 1995a; Lamb *et al.*, 1995), and the current study further confirms these findings. While THP-1 macrophage-mediated LDL oxidation was minimal in the absence of any ferrous ion supplementation (Figure 5.1), increasing concentrations of ferrous ions were associated with increasing rates of oxidation (Figure 5.2 & Figure 5.3). This requirement for transition metals has led to the suggestion that cells accelerate a metal-dependent oxidative process via the continuous reduction of these transition metals back to redox active forms (Garner & Jessup, 1996). Thus, it is not surprising that cell free LDL oxidation was similarly dependent on the supplemental iron concentration but that it simply proceeded at a slower rate than the corresponding cell-containing treatment (Figure 5.1 to Figure 5.3).

5.3.2 Protein Hydroperoxide Formation on LDL During Oxidation Mediated by Macrophages

5.3.2.1 Protein Hydroperoxide Formation and Lipid Peroxidation Are Correlated

THP-1 macrophages promoted the formation of 30-40 moles of protein hydroperoxides per mole of LDL, and this maximum level was observed whether the Ham's F10 medium was supplemented with 1.5µM or 3µM ferrous ions (Figure 5.2 & Figure 5.3). TBARS also peaked at a similar mole per mole scale, but the yield of conjugated dienes was approximately six to seven times larger. The disparity between these two markers of lipid peroxidation is explained by the fact that the TBARS assay detects malondialdehyde, an end-product of lipid peroxidation, while conjugated dienes provide a direct measure of conjugated lipid hydroperoxides (Esterbauer *et al.*, 1989). Using this more direct indication of lipid hydroperoxides, protein hydroperoxides can therefore be determined to account for 12-15% of the total LDL hydroperoxide content. This is comparable to the value of 16% calculated during copper-mediated LDL oxidation (Gieseg *et al.*, 2003) and is to be expected, especially considering that protein constitutes only 21.9% of the LDL by weight (Esterbauer *et al.*, 1992). It also confirms

for the first time that protein hydroperoxides are a significant product of THP-1 macrophage-mediated LDL oxidation.

Given sufficient time, similar levels of protein hydroperoxides were ultimately detected in the iron-containing cell free incubations (Figure 5.2 & Figure 5.3). Likewise, comparable quantities of protein hydroperoxides were generated on each LDL particle during both AAPH- and copper-mediated oxidation (Gieseg *et al.*, 2003). Such strong similarity between different oxidizing conditions indicates that apoB100's sensitivity to protein peroxidation is a general phenomenon. A common mechanism of apoB100 oxidation may exist, irrespective of the initial oxidizing agent.

The close parallel consistently observed between protein hydroperoxide formation and lipid peroxidation provides further support for a common mechanism of protein oxidation (Figure 5.6a & Figure 5.6b). This same correlation was previously noted during both AAPH- and copper-mediated LDL oxidation, and was further shown to remain undisturbed in the presence of either hydrophilic or hydrophobic antioxidants (Gieseg *et al.*, 2003). It has therefore been suggested that protein peroxidation and lipid peroxidation are linked, with protein hydroperoxides likely to be generated by a radical intermediate of the lipid peroxidation process. The theory appears equally relevant to cell-mediated LDL oxidation because the linkage between protein hydroperoxides and lipid peroxidation products was not broken by the incubation of THP-1 macrophages and LDL with a water soluble antioxidant like 7,8-NP (Figure 5.11). Moreover, the close linkage between the two ensured that the end of the protein hydroperoxide lag phase corresponded to the complete consumption of vitamin E (Figure 5.6e). This correlation occurs despite the fact that vitamin E is a lipid soluble antioxidant and, as such, is not expected to directly protect the apoB100.

The suggestion that protein oxidation can be initiated by lipid-derived radicals is certainly not new. In 1966 peroxidizing lipids were demonstrated to cause the loss of amino acids and of solubility in a variety of proteins (Roubal & Tappel, 1966a). The same authors also attributed the induction of protein polymerization to lipid peroxidation products (Roubal & Tappel, 1966b). Fluorescent products generated by a direct interaction between lipid peroxyl radicals and amino acids have been deteccted upon incubation of polypeptides with linoleoyl hydroperoxide (Fruebis *et al.*, 1992). More recently, an antibody has been described that is specific for protein damaged by lipid hydroperoxides and not aldehyde-modified or AAPH-damaged protein (Kim *et al.*, 1997). This same antibody also immunostained atherosclerotic lesions and LDL

oxidized by copper but did not cross-react with a native LDL control. Another group has demonstrated that the metal-catalyzed oxidation of PUFAs enhances carbonyl formation on pure proteins, with the extent of carbonyl formation increasing as the degree of lipid unsaturation increases (Refsgaard *et al.*, 2000). A strong correlation between LDL lipid peroxidation and apoB100 carbonyl formation in the presence of copper has, similarly, been attributed to protein oxidation induced by radical intermediates of lipid peroxidation (Yan *et al.*, 1997). Lipid-derived radicals are therefore likely to be responsible not only for protein hydroperoxide formation but also for production of the apoB100 carbonyls detected during macrophage-mediated LDL oxidation in the current study (Figure 5.6d).

5.3.2.2 A Comparison Between Protein Hydroperoxides and Additional Markers of Protein Oxidation

In comparison to the 30-40 moles of protein hydroperoxides generated per mole of LDL during THP-1 macrophage-mediated LDL oxidation, protein carbonyls peaked at eight moles/mole LDL (Figure 5.6d). A similar predominance of protein hydroperoxides over carbonyls has been noted in plasma after exposure to AAPH (Ling, 2003).

Specific markers of tyrosine oxidation were produced at even lower levels than carbonyls during LDL oxidation. Dityrosine remained undetectable, whether the LDL was incubated in the presence or absence of THP-1 macrophages, while the maximum PB-DOPA concentration only reached 0.29 moles/mole LDL (Figure 5.5). Thus, despite LDL containing 152 tyrosine residues (Esterbauer *et al.*, 1992), neither PB-DOPA nor dityrosine were found to be major products of macrophage-mediated or cell free LDL oxidation during the current incubation period. Other metal-dependent oxidation systems appear equally poor at inducing dityrosine formation on either LDL or alternative substrates, including tyrosine-containing tripeptides and lens proteins (Bruce *et al.*, 1999; Kato *et al.*, 2001; Gieseg *et al.*, 2003). In these studies, dityrosine formation only became significant upon the co-incubation of copper with hydrogen peroxide. PB-DOPA was at least detectable in a range of oxidizing systems, but concentrations remained low and were comparable to those recorded on LDL during THP-1 macrophage-mediated oxidation (Bruce *et al.*, 1999; Knott *et al.*, 2002; Gieseg *et al.*, 2003). While a range of incubation times was required to ultimately reach such

similar levels of PB-DOPA, this is likely to simply be due to the use of different LDL donors, oxidants, oxidant concentrations and LDL concentrations between the studies.

5.3.2.3 Protein Hydroperoxides are a General Consequence of Macrophage-Mediated LDL Oxidation

Lipid peroxidation products like conjugated dienes are widely recognized to form early during the oxidative modification of LDL. Thus, the correlation between these lipid peroxidation products and protein hydroperoxides (Figure 5.6b) not only supports the concept of protein being oxidized by lipid-derived radicals but also confirms that protein hydroperoxides are an early product of LDL oxidation. Combined with the significant quantities that have been detected during THP-1 macrophage-mediated LDL oxidation, protein hydroperoxides can therefore be considered a useful marker of apoB100 damage. However, it remained to be confirmed whether cell-mediated protein hydroperoxide formation was specific to the THP-1 macrophage cell line or whether it was a more general feature of all macrophage-mediated LDL oxidation. The finding that the primary HMDMs were also able to promote the formation of approximately 30 moles of protein hydroperoxides per mole of LDL (Figure 5.23a) confirms that the latter expectation is, indeed, correct. Protein hydroperoxides represent a general, and significant, consequence of macrophage-mediated LDL oxidation. Furthermore, the continued parallel between protein hydroperoxide and TBARS formation (Figure 5.23) suggests that a lipid-derived radical is likely to promote protein hydroperoxide formation during cell free oxidations and all macrophage-mediated LDL oxidations. Just as critically, these findings suggest that LDL will be vulnerable to protein hydroperoxide formation in the atherosclerotic plaque. There is some *in vivo* support for this claim because hydroxyleucine and hydroxyvaline are the break-down products of leucine hydroperoxide and valine hydroperoxide, respectively, and both have been detected in atherosclerotic samples (Fu *et al.*, 1998).

The variable rates of LDL oxidation observed in the presence of HMDMs contrast with the relative stability of THP-1 macrophage-mediated oxidation but do not invalidate any conclusions regarding protein hydroperoxide formation. This variability has been noted by other laboratories studying HMDM-mediated LDL oxidation (for example, Carpenter *et al.*, 1997) and, considering that each batch of HMDM cells is prepared using blood isolated from different donors, is to be expected.

5.3.3 Protein Hydroperoxides and Macrophage-Mediated Oxidation of BSA

Although macrophages are clearly capable of promoting significant protein hydroperoxide formation on LDL, this ability does not necessarily extend to other protein-containing compounds. Incubating a pure protein like BSA with THP-1 macrophages failed to produce any protein hydroperoxides detectable by the FOX assay (Figure 5.7 to Figure 5.9). BSA is susceptible to peroxidation when exposed to peroxyl radicals or irradiation (Duggan *et al.*, 2001) and, although albumin serves as an important transport protein in circulation, it has also been described as a sacrificial antioxidant (Frei *et al.*, 1988; Halliwell, 1988).

The absence of protein hydroperoxide formation in the current study occurred despite incubating under conditions known to be permissive for LDL oxidation, and despite testing a range of BSA concentrations and incubation times. Another group has reported a similar result when monitoring tryptophan levels in the presence of copper, with an early loss of tryptophan being measured on LDL but never BSA (Knott *et al.*, 2002). In an attempt to compensate for the known reducing activity of LDL, ascorbate was included in the BSA/copper incubation mixture, but even this failed to promote the loss of BSA's tryptophan residues.

There are several possible reasons for the lack of sensitivity exhibited by BSA towards cell-mediated protein hydroperoxide formation. Macrophage-mediated oxidation of LDL has been suggested to require an initial interaction between the lipoprotein and the LDL receptor (Aviram & Rosenblat, 1994). If correct, cell-mediated protein hydroperoxide formation on BSA could conceivably be prevented by the inability of the albumin protein to associate with the LDL receptor. The relevance of this theory is unclear because other groups have indicated that an interaction between LDL and its receptor is not essential for macrophage-mediated oxidation (Cathcart *et al.*, 1995; Tangirala *et al.*, 1996). Even if these later studies are correct, they do not rule out the possibility of a more non-specific interaction between macrophage cells and LDL's lipid component playing an important role in the promotion of oxidation.

It is also possible that the failure of THP-1 macrophages to promote the formation of BSA hydroperoxides is independent of any BSA-cell association and is, instead, due to a lack of a suitable interaction between BSA and transition metals. This is worthy of consideration because cells have been suggested to accelerate LDL oxidation by maintaining metals in redox active states (Garner & Jessup, 1996). Furthermore, copper

is considered to be a more potent mediator of oxidation than iron and this is, at least partly, due to its close interaction with the LDL particle (Lynch & Frei, 1995). Copper is known to bind albumin tightly (Kocha *et al.*, 1997) and, under certain circumstances, is certainly associated with the protein in its redox active form (Samocha-Bonet *et al.*, 2004). However, incubating BSA with copper alone does not induce a loss of tryptophan (Knott *et al.*, 2002) or a significant increase in carbonyl formation (Yan *et al.*, 1997). Additional reagents, such as those involved in Fenton chemistry, appear to be essential if copper-mediated BSA oxidation is to proceed. While the THP-1 macrophages represent a likely source of these additional reactants, Ham's F10 medium contains cupric ions at a concentration of only 0.01μM. It is expected that such low quantities would be unable to promote significant site-specific damage to the BSA. Iron also appears to be a relatively ineffectual oxidant under these circumstances because it binds only weakly to BSA (Kocha *et al.*, 1997). Thus, despite iron being present in supplemented Ham's F10 at a concentration of 4.5μM, the possibility of site-directed oxidation is virtually non-existent. Iron chelates, including citrate and nitrilotriacetate, can increase the oxidative potential of iron towards BSA (Ogino & Okada, 1995) but this finding is of little relevance under the conditions of the current study.

A third, and most likely, explanation for the lack of protein hydroperoxide formation on BSA arises from the theory that lipid-derived radicals are strong initiators of apoB100 oxidation. Consequently, the potential exists for these radicals to play an equally critical role during the metal-mediated oxidation of other proteins. As a pure protein like BSA contains no lipids, it would, by extension, lack susceptibility to any protein hydroperoxide formation initiated by radical intermediates of the lipid peroxidation chain reaction. Indirect support for this concept arises from a comparison of copper-mediated tryptophan loss on BSA and LDL. Under conditions designed to limit the difference between these treatments to the presence or absence of lipid, an early loss of tryptophan was observed only on LDL (Knott *et al.*, 2002). This led to the suggestion that tryptophan modification was a lipid peroxidation-dependent process. A more detailed analysis of copper-mediated BSA oxidation confirmed that carbonyl formation was also dependent on the presence of lipid (Yan *et al.*, 1997). This increase in carbonyls was observed whether using LDL, linoleic acid or a pre-formed hydroperoxide like tert-butyl hydroperoxide as the source of lipid. Copper was postulated to promote lipid peroxidation and hydroperoxide decomposition, with some of the resulting lipid-derived radicals being responsible for initiating BSA oxidation.

5.3.4 Effect of 7,8-NP on Macrophage-Mediated LDL Oxidation

5.3.4.1 7,8-NP Inhibits Protein Hydroperoxide Formation During Macrophage-Mediated LDL Oxidation

Given the reactivity of protein hydroperoxides, and their potential to cause additional damage, it was important to investigate whether their formation could be inhibited during macrophage-mediated LDL oxidation. 7,8-NP has previously been shown to inhibit LDL oxidation under cell free conditions, with Gieseg *et al.* (1995) noting a clear protection against lipid peroxidation and vitamin E loss in the presence of copper ions. More recently, 7,8-NP has been confirmed to prevent not only lipid peroxidation but also protein hydroperoxide formation on LDL during AAPH- and copper-mediated oxidation (Gieseg *et al.*, 2003). The peroxidation of LDL lipid in the presence of both THP-1 monocytes and THP-1 macrophages is also efficiently inhibited by this pteridine (Gieseg & Cato, 2003). 7,8-NP's protection against THP-1 macrophage-mediated damage is now shown to extend to the protein component of LDL. Protein hydroperoxide formation was inhibited in a concentration-dependent manner that paralleled the prevention of lipid peroxidation, as determined by TBARS (Figure 5.10). This concentration-dependent effect resulted in the complete protection of both the lipid and protein components of LDL by 30μM 7,8-NP. In comparison, THP-1 monocyte-mediated TBARS formation was completely inhibited on LDL in the presence of only 5μM 7,8-NP (Gieseg & Cato, 2003). Considering that macrophages are more potent inducers of LDL oxidation than the corresponding monocytes (Fuhrman *et al.*, 2004), the requirement for a higher 7,8-NP concentration is not unexpected.

In addition to preventing damage to the protein and lipid components of LDL, this pteridine also spared the loss of large quantities of vitamin E (Figure 5.10c). Thus, 7,8-NP can be considered to significantly protect the entire LDL particle from THP-1 macrophage-mediated oxidative attack. A concentration of 30μM 7,8-NP was once again shown to provide maximal protection, promoting the retention of approximately 80-85% of the initial vitamin E concentration. The inability of 7,8-NP to completely protect vitamin E has also been noted during earlier LDL oxidation studies (Gieseg & Cato, 2003) and may be due to a scavenging competition between the two antioxidants for lipid peroxyl radicals. Indeed, 7,8-NP has previously been suggested to protect LDL against AAPH- and copper-mediated oxidation by directly competing with vitamin E for these lipid peroxyl radicals (Gieseg *et al.*, 1995).

5.3.4.2 Mechanism of 7,8-NP-Mediated Protection

Time course data indicated that 7,8-NP was able to extend the lag phase of both TBARS and protein hydroperoxide formation in a concentration-dependent manner, while having minimal effect on oxidation rates during the propagation phase (Figure 5.11). This agrees with the kinetics observed, in the presence of 7,8-NP, during both AAPH- and copper-mediated LDL lipid and protein oxidation (Gieseg *et al.*, 1995; Gieseg *et al.*, 2003). However, this similarity does not extend to 7,8-NP's effect on vitamin E consumption. While 7,8-NP merely slowed the rate of vitamin E loss during copper-mediated LDL oxidation (Gieseg *et al.*, 1995), it was able to spare vitamin E almost entirely during the early stages of THP-1 macrophage-mediated LDL oxidation (Figure 5.11c). This sparing effect was concentration-dependent, with increasing quantities of 7,8-NP completely protecting the vitamin E for increasing periods of time. The subsequent loss of vitamin E began slowly in some instances but, by the final stages, occurred at rates largely independent of the starting 7,8-NP concentration.

The different effects of 7,8-NP on copper-mediated and THP-1 macrophage-mediated vitamin E loss are most likely due simply to the dissimilar rates of oxidation between each of the oxidizing systems and will be discussed in further detail below. Nevertheless, it remained possible that the 7,8-NP was inhibiting THP-1 macrophage-mediated LDL oxidation by mechanism(s) independent of its chain-breaking scavenging of the LDL lipid peroxyl radicals. Perhaps this pteridine was able to prevent macrophage-mediated LDL oxidation by some, as yet unidentified, direct interaction with the cells. Gieseg & Cato (2003) certainly reported a need to pre-incubate the 7,8-NP with THP-1s for consistent protection. By contrast, the AAPH and copper oxidizing systems required a pre-incubation of the 7,8-NP with LDL (Gieseg *et al.*, 1995).

i) 7,8-NP and TPMET Activity

TPMET is suggested to play a critical role in macrophage-mediated LDL oxidation (Baoutina *et al.*, 2001b) and, as such, it was possible that 7,8-NP could be exerting its inhibitory effect via an interaction with this system. Between 75-90% of ferricyanide reduction could generally be attributed to THP-1 macrophage TPMET activity, with the remainder being due to cell-secreted reductants (eg. Figure 5.16 & Figure 5.17). This proportion is in close agreement with the over 81% of ferricyanide reducing activity

attributed to the TPMET system during an earlier study involving THP-1 macrophages (Baoutina *et al.*, 2001b). By contrast only 40-60% of copper reducing activity could be accounted for by TPMET (eg. Figure 5.16 & Figure 5.17). The seemingly higher proportion of copper reducing activity in the cell-conditioned medium may be due to the ability of BCS (an assay reagent) to alter the redox potential of copper, thereby shifting the equilibrium towards greater cupric ion formation (Sayre, 1996). While this can affect the measured concentrations of reduced copper, it does not detract from studies investigating 7,8-NP's influence on the cellular reducing activity.

It soon became apparent that a pre-incubation with 50μM 7,8-NP had no detectable impact on the subsequent activity of either the cell-secreted reductants or the TPMET system (Figure 5.16). This was consistently observed by both reduction assays and occurred despite the fact that 50μM 7,8-NP is known to significantly inhibit THP-1 macrophage-mediated LDL oxidation (Figure 5.10 & Figure 5.11). The possibility that TPMET activity is inhibited only when continuously exposed to 7,8-NP cannot be completely ruled out. Such a study is not feasible due to the strong interference experienced by both reduction assays in the presence of micromolar concentrations of 7,8-NP. Nevertheless, the probability of any significant interaction between 7,8-NP and TPMET is extremely unlikely because the continuous exposure of THP-1 macrophages to IFN-γ generated nanomolar quantities of pterin (Figure 5.18) but failed to modulate TPMET activity (Figure 5.17). While another study did observe an IFN-γ-mediated inhibition of TPMET activity (Sun *et al.*, 1996), this disparity may simply be due to the use of the HL60 cell line rather than THP-1 macrophages.

ii) 7,8-NP and Radical Scavenging

The inability of 7,8-NP to inhibit TPMET activity ultimately suggests that, rather than modulating macrophage activity, 7,8-NP most likely provides protection against the oxidative modification of LDL by scavenging radicals. Further support for this type of antioxidant activity arises from the fact that, during THP-1 macrophage-mediated LDL oxidation, the 7,8-NP progressively oxidizes to 7,8-XP (Figure 5.12). 7,8-NP loss even correlates to the phases of LDL oxidation, with the lag phase ending and the propagation phase begining only once most of the 7,8-NP has been consumed.

Although the scavenging of LDL lipid peroxyl radicals provides one explanation for these kinetics, it remained possible that 7,8-NP's scavenging activity was instead due to

a reaction with cell-associated radicals. Membrane-derived peroxyl radicals represent one potential scavenging target because the incubation of cells with ferrous ions, as in the current study, has been shown to promote lipid peroxidation on a range of monocyte and macrophage cells (Fuhrman *et al.*, 1994; Gieseg *et al.*, 2001b). This peroxidation displayed an iron-dose dependence and, while iron levels in the supplemented Ham's F10 are 20-50% of those tested by other studies, some lipid peroxidation in the current study is still feasible. Just as critically, cellular lipid peroxides have been found to enhance LDL oxidation (Fuhrman *et al.*, 1994; Keidar *et al.*, 1995; Fuhrman *et al.*, 1997). Thus, it can be envisaged that by protecting macrophages from iron-induced peroxidation, 7,8-NP could indirectly protect the LDL particle from oxidative modification. This theory is worthy of consideration because 7,8-NP has previously been found to prevent iron-induced lipid peroxidation on U937 monocytes (Gieseg *et al.*, 2001b). However, the kinetics of 7,8-NP consumption and 7,8-XP formation in the presence of THP-1 macrophages do not provide strong support for an interaction between 7,8-NP and any cell peroxides that may exist under the conditions of the current study (Figure 5.14). If 7,8-NP was scavenging cell-derived radicals, the oxidation rate for this pteridine should increase dramatically upon incubation with THP-1 macrophages. Instead, the loss of 7,8-NP in the absence of any cells or LDL was relatively slow, and the addition of THP-1 macrophages did not speed up this oxidation. By contrast, co-incubation with LDL resulted in an increased rate of loss for 7,8-NP, and the addition of THP-1 macrophages to this LDL/pteridine incubation mixture enhanced 7,8-NP oxidation even further. This latter result is to be expected because cells are known to significantly enhance LDL oxidation compared to the corresponding cell free conditions. The consumption of 7,8-NP therefore appears to be dependent upon the presence of LDL, and the oxidizing capacity of the environment, rather than the specific presence of the THP-1 macrophages themselves.

Given the above findings, it appears that the distinct trends of vitamin E oxidation observed in the current study and the Gieseg *et al.* (1995) study may simply be due to the different oxidizing capacities of the two environments. Sources of this difference could range from the degree of oxygen exposure to the use of alternative oxidants and incubation media. Copper-mediated cell free LDL oxidation occurred in PBS, subjected to continuous swirling in a flask. Cell-mediated LDL oxidation experiments involved incubation in Ham's F10, using plates that were exposed to a constant environment of 5% carbon dioxide and little motion. Whatever the cause of the different oxidizing

capacities, simple rate calculations indicate that 5μM copper was able to support the generation of approximately 610 mmoles of protein hydroperoxides per mole of LDL per minute during the propagation phase (Gieseg *et al.*, 2003). By contrast, only 110 mmoles of protein hydroperoxides were formed per mole of LDL every minute during THP-1 macrophage-mediated LDL oxidation (Figure 5.11). In the absence of 7,8-NP, vitamin E was consumed at a rate of 300 mmole per mole of LDL per minute during copper oxidation (not 0.5nM/min as stated in Gieseg *et al.*, 1995). Only 18 mmoles were oxidized per mole of LDL every minute during the linear phase of cell-mediated vitamin E oxidation (Figure 5.11). Not only do the oxidation rates differ significantly between these two studies but the ratio of 7,8-NP to vitamin E also shows large variability. The current study is associated with an initial antioxidant ratio of 136 moles of 7,8-NP per mole of vitamin E (Figure 5.13b), but Gieseg *et al.* (1995) used a significantly lower ratio of three to four moles of 7,8-NP per mole of vitamin E.

7,8-NP and vitamin E have been reported to scavenge peroxyl radicals at comparable rates (Oettl *et al.*, 1997) and thus, given the differences above, it appears that 7,8-NP is able to more effectively compete for radicals and spare the vitamin E from oxidation under conditions associated with the cell studies rather than the copper studies. Indeed, 20μM 7,8-NP was observed to decrease almost immediately during cell-mediated LDL oxidation while a delay in vitamin E loss was clearly apparent (Figure 5.13b). Only after the loss of sufficient quantities of 7,8-NP did vitamin E also begin to scavenge the lipid peroxyl radicals and become oxidized.

While all tested concentrations of 7,8-NP showed a protective effect against THP-1 macrophage-mediated oxidation, the lower concentrations generally portrayed this antioxidant effect more efficiently (Figure 5.10 & Figure 5.11). Whether 7,8-NP may be exerting a slight pro-oxidant effect by the later stages of incubation with 50μM 7,8-NP is unclear and was not further investigated. Nevertheless, the potential for 7,8-NP to promote LDL oxidation under various conditions has been noted (Herpfer *et al.*, 2002; Greilberger *et al.*, 2004). The result also bears similarities to a study by Herpfer *et al.* (2002). They observed an antioxidant effect in the presence of 150μM 7,8-NP during copper-mediated LDL oxidation, but this was weaker compared to the effect of 100μM 7,8-NP.

5.3.4.3 7,8-NP Inhibits Both THP-1 Macrophage- and HMDM-Mediated LDL Oxidation

Regardless of the potential for such pro-oxidant activity at higher pteridine concentrations and/or later stages of incubation, the overall activity of 7,8-NP during THP-1 macrophage-mediated LDL oxidation is clearly antioxidant in nature. This protection was confirmed to span the entire LDL particle and was not restricted to a single cell line. 7,8-NP was able to prevent protein hydroperoxide formation, lipid peroxidation and vitamin E loss in the presence of the primary HMDMs as well (Figure 5.24).

One point of difference between the two cell types was the antioxidant concentration required to completely inhibit cell-mediated LDL oxidation. While 30μM 7,8-NP was sufficient for THP-1 macrophages, a minimum concentration of 100μM 7,8-NP was required in the presence of HMDMs. The latter experiment required a longer incubation time, but this is unlikely to completely account for the differing efficacy of 7,8-NP in the two cell systems. Inter-experimental variability meant that different HMDM preparations required different incubation times. However, there was no correlation to the corresponding concentration of 7,8-NP required for complete protection.

The apparently weaker potency of 7,8-NP during HMDM-mediated LDL oxidation may be better explained by structural differences betweem these two macrophage types. Unlike THP-1 macrophages, HMDM cells are relatively large and lipid-loaded. Cells are generally considered to promote LDL oxidation by maintaining transition metals in their redox active states. However, the possibility of an interaction between the LDL and macrophages does exist (Tangirala *et al.*, 1996). Non-specific interactions between LDL lipid and macrophage lipid may be more pronounced in the lipid-loaded HMDMs and this could potentially hinder 7,8-NP's scavenging of LDL lipid peroxyl radicals. As discussed in section 3.3.3, the lipid-loaded phenotype may also cause 7,8-NP itself to interact or partition with HMDMs. The interaction could occur in such a way that the 7,8-NP would be unable to scavenge LDL lipid peroxyl radicals as efficiently as it does in the presence of THP-1 macrophages.

While these issues remain to be clarified, one thing is apparent. The finding that 7,8-NP can inhibit protein hydroperoxide formation during both THP-1 macrophage- and HMDM-mediated LDL oxidation may have far-reaching and beneficial consequences. This is because protein hydroperoxides are not inert and, once formed, can consequently cause additional damage to a range of substrates including DNA (Gebicki & Gebicki,

1999; Luxford *et al.*, 1999), antioxidants (Simpson *et al.*, 1992; Fu *et al.*, 1995) and proteins (Gebicki *et al.*, 1995; Davies, 1996a; Soszynski *et al.*, 1996; Hampton *et al.*, 2002; Morgan *et al.*, 2002). Thus, by inhibiting protein hydroperoxide formation, 7,8-NP not only protects the LDL particle but also prevents any adverse reactions that may subsequently occur between the reactive protein hydroperoxide moiety and other biomolecules.

5.3.5 Effect of IFN-γ on Macrophage-Mediated LDL Oxidation

5.3.5.1 IFN-γ-Stimulated THP-1 Macrophages Do Not Significantly Inhibit LDL Oxidation

The concentrations of exogenously-supplied 7,8-NP required for effective inhibition of macrophage-mediated LDL oxidation are significantly higher than the nanomolar quantities released in cell culture upon stimulation of macrophages with IFN-γ (Figure 5.18; Figure 5.25; Weiss *et al.*, 1992; Weiss *et al.*, 1999; Gieseg & Cato, 2003). While this has raised some questions regarding the *in vivo* relevance of such findings, it is nevertheless important to still consider whether the continuous release of 7,8-NP would permit more efficient antioxidant activity than a single, bolus addition at the start of an experiment. Furthermore, stimulation with IFN-γ alone may not promote maximal pterin production in macrophages. Several biomolecules, including TNFα and LPS, have previously been shown to enhance neopterin production when co-incubated with IFN-γ (Werner-Felmayer *et al.*, 1990; Werner-Felmayer *et al.*, 1995). Although the concentration of neopterin generated under these conditions remained in the nanomolar range, it is possible that additional, but as yet unidentified, stimulants or combinations of stimulants may ultimately raise production further. One research group has also questioned whether contact with other cells may be critical for optimizing pterin synthesis (Wirleitner *et al.*, 2001; Enzinger *et al.*, 2002a). *In vivo*, concentrations of up to 0.1μM neopterin have certainly been reported in the serum of HIV (Fuchs *et al.*, 1994) and atherosclerosis (Tatzber *et al.*, 1991) patients while, more recently, neopterin has been found to range from nanomolar concentrations up to 1.28μM in inflammatory sites (chapter 6). Thus, the potential exists for levels to be even higher in microenvironments immediately adjacent to the pterin-synthesizing macrophages. Similarly, although intracellular pterin has been detected in the pmole/mg protein range after stimulation with IFN-γ (Figure 5.18; Figure 5.25; Werner *et al.*, 1989; Werner-

Felmayer *et al.*, 1990), it is possible that a more highly concentrated microenvironment may exist within the macrophage cells.

Given all this, it was deemed worthwhile to investigate whether IFN-γ exerted any influence on macrophage-mediated LDL oxidation. While previous studies have indicated that IFN-γ can inhibit LDL oxidation in the presence of mouse macrophages (Fong *et al.*, 1994), this finding is of little relevance to the current study. The mouse macrophage-derived antioxidant activity was attributed to production of nitric oxide (Jessup & Dean, 1993; Niu *et al.*, 2000), but human macrophages synthesize 7,8-NP rather than nitric oxide upon stimulation with IFN-γ (Werner *et al.*, 1990). Pre-incubating THP-1 macrophages in RPMI with 500U/ml IFN-γ for 48 hours certainly elevated neopterin levels (Figure 5.18). However, a subsequent incubation in ferrous-supplemented Ham's F10, containing LDL and fresh IFN-γ, failed to inhibit LDL oxidation compared to an unstimulated THP-1 macrophage control (Figure 5.19). This lack of protection was not due to an LDL- or iron-mediated inhibition of 7,8-NP synthesis. Pterin levels were largely unaffected by incubation with the lipoprotein and, although iron has been reported to inhibit neopterin production, this generally requires significantly higher concentrations than those used during LDL oxidation studies (Weiss *et al.*, 1992).

The lack of any significant IFN-γ-mediated protection contrasts with results from another study (Gieseg & Cato, 2003). THP-1 macrophages stimulated with IFN-γ appeared to slow the rate of vitamin E loss on LDL in that investigation, though a significant difference in the extent of lipid peroxidation was apparent at only one time point. The slower rate of THP-1 macrophage-mediated LDL oxidation associated with this earlier study likely aided detection of such low levels of inhibition. As a consequence, experimental modifications were introduced to the current investigation in an attempt to create more optimal conditions for detecting any IFN-γ-mediated effect. A 48 hour pre-incubation of THP-1 macrophages in Ham's F10, rather than RPMI, not only slowed the rate of LDL oxidation (Figure 5.21) but also ensured that no 7,8-NP released into the medium during pre-incubation would be lost at the start of the LDL incubation. Although protein hydroperoxide and TBARS formation did appear slightly slower in the IFN-γ treatment under these conditions, this difference never reached significance and was not supported by the vitamin E data (Figure 5.21). Furthermore, the trend was not consistently observed, with some experiments showing rates of reaction that were identical to those observed in the absence of IFN-γ. The reason for

the continued disparity with the Gieseg & Cato (2003) study is unclear but the possibility of some unidentified difference existing between the two separate stocks of THP-1s cannot be completely ruled out. The plausibility of this concern is highlighted by the strong effect of IFN-γ in the presence of another macrophage type, the primary HMDMs.

5.3.5.2 IFN-γ-Stimulated HMDMs Strongly Inhibit LDL Oxidation

In contrast to both THP-1 macrophage studies (Figure 5.21; Gieseg & Cato, 2003), IFN-γ-stimulated HMDMs were associated with an intense antioxidant action (Figure 5.27). Protection extended to the entire LDL particle, with vitamin E loss, protein hydroperoxide formation and lipid peroxidation all being inhibited. This inhibition was generally of a sufficient strength that the IFN-γ treatment remained in the lag phase after LDL from the unstimulated HMDM control had entered the decomposition phase. Just as critically, HMDMs incubated with IFN-γ lost no significant viability or metabolic activity compared to controls (Figure 5.26). Thus, the lack of oxidation was real and not an artefact of the experimental system. Another study has also noted this IFN-γ-mediated inhibition in the presence of HMDMs and copper but made no attempt to explain the results (Hurt-Camejo *et al.*, 1992).

Although the IFN-γ-mediated protection against LDL oxidation was always detected, the extent of the inhibition did show some variability from one HMDM donor to the next. While variable rates of LDL oxidation had previously been observed in the presence of unstimulated HMDMs (section 5.3.1), it failed to correlate with the IFN-γ-stimulated HMDM variability. As a result, this latter variability appears to be caused predominantly by differences in donor responsiveness towards IFN-γ rather than any differences in the inherent capacity of unstimulated HMDMs to promote LDL oxidation.

i) Mechanism of IFN-γ-Mediated Protection

Despite the fact that IFN-γ induced THP-1 macrophages and HMDMs to synthesize similar quantities of neopterin (Figure 5.18 & Figure 5.25), only one of these cells was able to significantly inhibit LDL oxidation in the presence of this cytokine. Such a clear lack of correlation between neopterin production and antioxidant activity raised serious doubts about 7,8-NP's role in the inhibitory process. Though not definitively studied,

IFN-γ has been observed to cause an increase in the area of a number of other peaks detected during HPLC pterin analysis (data not shown). Thus, the possibility exists that another pterin could be exerting an antioxidant effect.

It must also be remembered that IFN-γ is a multifunctional cytokine and has the potential to modulate HMDM-mediated LDL oxidation by a number of pterin-independent mechanisms. As an example, one earlier study also observed an inhibition of LDL oxidation in the presence of IFN-γ-stimulated HMDMs (Christen *et al.*, 1994). This effect was attributed to IFN-γ's upregulation of indoleamine 2,3-dioxygenase (IDO), a tryptophan degrading enzyme that ultimately results in the generation of 3-hydroxyanthranilic acid (3-HAA). Although 3-HAA is a potent antioxidant of LDL oxidation (Christen *et al.*, 1990; Thomas *et al.*, 1996b), it is not relevant to the current study. 3-HAA formation is dependent on the supplementation of Ham's F10 with 75μM tryptophan (Christen *et al.*, 1994), but the Ham's F10 used in the current study contains only an unsupplemented tryptophan concentration of 2.94μM.

While the exact source of the IFN-γ-mediated inhibition remains to be determined, it does not appear to be an integral component of the HMDMs but is, instead, a secreted substance. Medium conditioned by IFN-γ-stimulated HMDMs strongly inhibited LDL oxidation, even in the presence of 3μM cupric ions (Figure 5.28). By contrast, unstimulated HMDM-conditioned medium supported LDL oxidation at a rate identical to the one observed under cell free conditions. This indicates that the pro-oxidant activity of the HMDMs is strongly associated with the cells themselves but the antioxidant effect is largely, if not entirely, due to cellular secretions. The strength of the inhibition caused by cell-secreted products provides further evidence against an antioxidant role for 7,8-NP during HMDM-mediated LDL oxidation. The 100μM 7,8-NP concentration previously shown to be required for complete protection (Figure 5.24) is several orders of magnitude larger than the nanomolar quantities of pterin released into the medium by IFN-γ-stimulated HMDMs (Figure 5.25).

In addition to stimulating 7,8-NP and 3-HAA production, IFN-γ has also been reported to reduce lipoxygenase activity in macrophages (Fong *et al.*, 1994; Folcik *et al.*, 1997). Just like 7,8-NP and 3-HAA, this activity is also not expected to account for the IFN-γ-mediated inhibition of LDL oxidation observed in the current study. The conditioned medium from unstimulated macrophages possessed no pro-oxidant activity that would be susceptible to such down-regulation in the presence of IFN-γ (Figure 5.28). Furthermore, the unidentified antioxidant is thought to be secreted from HMDMs

while 15-lipoxygenase is associated with an intracellular location (Cathcart & Folcik, 2000).

IFN-γ must therefore be inducing the protective effect via a pathway independent of the inhibition of lipoxygenase activity and the production of 3-HAA or 7,8-NP, possibly one involving the release of a metal-chelating agent. Metal sequestration by macrophage cells has certainly been described (Kritharides *et al.*, 1995a; van Reyk *et al.*, 1999). While some of this activity was attributed to sequestration within the cells, macrophages were also suggested to provide a more antioxidative environment by directly modifying the medium (van Reyk *et al.*, 1999). This modification could occur by numerous mechanisms, including the secretion of metal chelators.

Whatever the source of the antioxidant activity, its release must be enhanced in IFN-γ-stimulated HMDMs but not in IFN-γ-stimulated THP-1 macrophages if it is to conform to the findings of the current study. Ceruloplasmin represents a classic example of this, with synthesis being upregulated strongly by IFN-γ in HMDMs but only weakly in THP-1s (Mazumder *et al.*, 1997). As a copper carrier that also possesses ferroxidase activity (Fox *et al.*, 2000), this protein initially seems like an ideal source of the IFN-γ-mediated HMDM antioxidant activity. However, when incubated with cells or reducing agents released by cells, ceruloplasmin has also been shown to promote LDL oxidation (Ehrenwald *et al.*, 1994; Ehrenwald & Fox, 1996; Fox *et al.*, 2000). While beyond the scope of the current study, this issue could be further investigated by confirming the relative levels of ceruloplasmin present in the conditioned medium isolated from each cell type. As IFN-γ is known to enhance ceruloplasmin production by 20-fold in the U937 cell line (Mazumder *et al.*, 1997), a comparative study of LDL oxidation in the presence of IFN-γ-stimulated U937s could provide additional, indirect evidence regarding ceruloplasmin's relevance to the oxidative process. More generally, the potential for any HMDM-derived metal chelator to inhibit LDL oxidation could be investigated by subjecting LDL to a metal-independent form of oxidative stress. The peroxyl radical generator, AAPH, represents just such an oxidant and would need to be incubated with the LDL in IFN-γ-stimulated HMDM-conditioned medium. A lack of protection under these experimental conditions would provide reasonable support for the existence of a HMDM-derived metal chelator. By contrast, the detection of antioxidant activity in the presence of a metal-independent oxidant would not necessarily deny an inhibitory role for metal chelators during LDL oxidation. The range of compounds secreted by IFN-γ-stimulated macrophages may simply serve as

alternative substrates for radical attack and, as such, represent sacrificial antioxidants of the metal-independent oxidation. Care must clearly be taken when interpreting results from such experiments. If protein released from IFN-γ-stimulated macrophages served as a sacrificial antioxidant, it is expected to be more highly concentrated in the medium conditioned by IFN-γ-treated HMDMs than in media conditioned by either control HMDMs or IFN-γ-treated THP-1 macrophages. Gel electrophoresis provides one means to investigate the existence of such differences. Ultimately, it must also be recalled that IFN-γ is a multifunctional cytokine and, as such, could be inhibiting the HMDM-mediated LDL oxidation by multiple pathways.

Although the current investigation found no evidence of antioxidant activity associated with the 7,8-NP produced by IFN-γ-stimulated macrophages, the possibility of an effect *in vivo* cannot be eliminated. Atherosclerosis develops over a period of decades, and so, with the slower rates of oxidation that are expected to occur *in vivo*, the low but constant production of pterin may have beneficial consequences in such an environment.

5.3.6 Summary

Macrophage-mediated LDL oxidation may play a critical role in the development of atherosclerosis. While many studies have investigated such processes, most have focussed on lipid rather than protein peroxidation. The finding that protein hydroperoxide formation was promoted on the LDL during both THP-1 macrophage- and HMDM-mediated oxidation indicates that this process is a general feature of all macrophages rather than merely being restricted to one cell type. With approximately 30-40 protein hydroperoxides being formed per LDL particle at the peak of oxidation, this moiety can also be considered a significant consequence of macrophage-mediated oxidation. It was produced in greater quantities than other markers of protein oxidation, including carbonyls and PB-DOPA. Protein hydroperoxide generation on LDL has previously been suggested to involve lipid-derived radicals, and the close parallel between protein hydroperoxide formation and lipid peroxidation in the current study provides support for this theory. The close parallel further indicates that, like lipid peroxidation, protein peroxidation is an early product of LDL oxidation. All this suggests that protein hydroperoxides are likely to be generated on LDL in the atherosclerotic plaque.

Given the reactivity but relative stability of protein hydroperoxides, this moiety has the potential to cause additional damage and may have important implications for atherosclerosis. However, there is also the potential to prevent protein hydroperoxide generation. An incubation with the pteridine, 7,8-NP, inhibited both THP-1 macrophage- and HMDM-mediated protein hydroperoxide formation on LDL. This occurred in a concentration-dependent manner that paralleled the inhibition of lipid and vitamin E oxidation. Thus, 7,8-NP has the capacity to protect the entire LDL particle, including the protein component, from cell-mediated oxidative attack. While the TPMET system is suggested to be important for macrophage-mediated LDL oxidation (Baoutina *et al.*, 2001b), 7,8-NP does not protect the LDL by modulating this activity. Instead, it appears to act as a chain-breaking antioxidant by scavenging lipid peroxyl radicals in direct competition to the LDL's vitamin E. A similar mechanism has previously been attributed to 7,8-NP during studies of copper- and AAPH-mediated LDL oxidation (Gieseg *et al.*, 1995; Gieseg *et al.*, 2003). In support of this radical scavenging activity, 7,8-NP was able to extend the lag phase while having a minimal impact on the rate of macrophage-mediated LDL oxidation during the propagation phase. Such observations can be accounted for by the kinetics of 7,8-NP consumption, which was generally completely oxidized to 7,8-XP by the end of the lag phase. 7,8-NP oxidation was also shown to be dependent upon the presence of LDL, and the oxidizing capacity of the environment, rather than the THP-1 macrophages themselves.

Unlike IFN-γ-stimulated THP-1 macrophages, IFN-γ-stimulated HMDMs were found to be potent inhibitors of macrophage-mediated LDL oxidation. This contrast was observed even though IFN-γ induced both macrophages to produce similar quantities of pterin. Just as critically, pterin in the supernatant was only detected at nanomolar levels. Despite a single dose of 7,8-NP at these concentrations being insufficient to inhibit LDL oxidation, medium conditioned by IFN-γ-treated HMDMs retained the potent antioxidant activity. This all suggests that, while the 7,8-NP produced by IFN-γ-stimulated macrophages did not play a significant role in preventing LDL oxidation in the current study, macrophages can nevertheless exhibit antioxidant behaviour under certain conditions. The differential effect of IFN-γ on the ability of each macrophage cell type to oxidize LDL also provides a renewed warning against extrapolating directly from cell lines to primary cells.

6 Neopterin and Markers of Oxidation in Inflammatory Environments

6.1 Introduction

Neopterin is a product of IFN-γ-stimulated macrophages and, as such, has long been associated with macrophage activation and inflammation. This relationship has highlighted the potential for neopterin to serve as a marker of disease activity. Indeed, the correlation between neopterin concentration and disease severity/extent has been investigated for numerous infections and diseases, including coronary artery disease (CAD).

Hospitalized but not non-hospitalized patients with atherosclerosis were shown to possess significantly elevated quantities of neopterin compared to healthy controls (Tatzber *et al.*, 1991). Even the hospitalized subjects could be further stratified, with those exhibiting signs of peripheral occlusions appearing to have higher serum neopterin levels than the coronary occlusion patients (Tatzber *et al.*, 1991; Erren *et al.*, 1999). More comprehensive studies identified a direct correlation between neopterin concentrations in the serum and the extent of atherosclerosis. This association remained whether monitoring disease progression in the coronary arteries (Gurfinkel *et al.*, 1999), or in the carotid artery of a general atherosclerotic population (Weiss *et al.*, 1994) and an atherosclerotic subpopulation of haemodialysis patients (Erten *et al.*, 2005). Other groups have identified a correlation between serum neopterin levels and the number of complex coronary stenoses in patients with either unstable angina (Garcia-Moll *et al.*, 2000a) or non-ST segment elevation acute coronary syndromes (Avanzas *et al.*, 2004a). These same studies, however, failed to find an association between serum neopterin and disease extent when measuring other angiographic markers of atherosclerotic progression like vessel score. Detecting these correlations therefore appears to be dependent on additional factors that range from sample size, to the method for determining CAD severity, and even whether neopterin is expressed before or after normalization against creatinine.

In another negative finding, Schumacher *et al.* (1992) found no difference in neopterin levels between males with either minor or severe CAD. A more comprehensive study by the same group several years later reaffirmed this lack of

significance, though it was acknowledged that the use of a larger sample size did begin to uncover a trend of increasing serum neopterin with increasing CAD (Schumacher *et al.*, 1997). It was also acknowledged that neopterin was significantly more concentrated in the serum of subjects suffering from acute myocardial infarction compared to both healthy controls and patients with stable CAD. This distinction between acute and chronic syndromes was contradicted by another group (Auer *et al.*, 2001), but the Auer *et al.* (2001) result appears to be an exception to the general trend. All other studies support the Schumacher *et al.* (1997) findings of serum neopterin levels generally being higher in unstable or acute conditions than in stable but chronic forms of CAD (Gupta *et al.*, 1997a; Garcia-Moll *et al.*, 2000b; Ilhan *et al.*, 2005). Even neopterin levels in urine have been shown to increase significantly in the week following an acute myocardial infarction (Melichar *et al.*, 1994). IFN-γ has been detected in atherosclerotic plaque (Geng *et al.*, 1995; Frostegard *et al.*, 1999), and sites of plaque rupture/instability are associated with high concentrations of macrophages (Bjorkerud & Bjorkerud, 1996). Given the mechanism of pterin synthesis, these sites would therefore also be expected to represent regions of high neopterin content. Thus, the association with macrophage activity provides a simple explanation for the correlations between neopterin and adverse cardiac events.

The potential also exists for neopterin to differentiate between distinct types of acute and unstable coronary syndromes. Patients diagnosed with a non-Q-wave acute myocardial infarction have been associated with higher quantities of serum neopterin than those diagnosed with unstable angina (Gurfinkel *et al.*, 1999). Another study classified 32 women, all diagnosed with unstable angina, into two sub-groups (Garcia-Moll *et al.*, 2000b). High serum neopterin levels were associated with women who subsequently experienced additional adverse cardiac events. Conversely, lower neopterin serum concentrations tended to be observed in women free of such events.

Several additional studies have evaluated the prognostic value of neopterin further. This pteridine was shown to be predictive of adverse cardiac events in patients who had previously suffered a non-Q-wave myocardial infarction (van Haelst *et al.*, 2003) or who were diagnosed with hypertension but did not exhibit signs of obstructive CAD (Avanzas *et al.*, 2004b). Likewise, higher serum neopterin levels in patients with chronic stable angina were associated with a higher risk of adverse cardiac events (Avanzas *et al.*, 2005). This ability to predict disease progression in such patients was so strong that it remained significant even after adjusting for a range of other potential

risk factors, including the presence of complex lesions (Zouridakis *et al.*, 2004). Serum neopterin levels were also shown to be predictive of mortality one year after the onset of critical limb ischemia in patients with peripheral atherosclerosis (Barani *et al.*, 2005).

Although the above associations are all statistically significant, it should be noted that the differences in serum neopterin between the control and diseased groups are often relatively small. Concentrations may even overlap between the two groups, thereby reducing the effectiveness of neopterin as an efficient marker of disease activity when assessing individuals rather than populations. What these studies all clearly show, however, is the prominence of immune activation during atherosclerosis. By contrast, they provide no evidence either for or against an *in vivo* antioxidant action of 7,8-NP (the unoxidized form of neopterin). This is because the previous studies only measured pterin in the serum, but the nanomolar (or ng/ml) quantity detected here is, of course, likely to be highly diluted compared to neopterin at the original inflammatory site of formation. Such a distinction is important because the potential for 7,8-NP to act as an antioxidant *in vivo* will strongly depend upon its concentration at these sites of oxidant attack. Admittedly, one study did attempt to gain a more accurate idea of neopterin concentrations associated with stable CAD by collecting blood directly from the aortic root and coronary sinus (Tanaka *et al.*, 2004). Levels remained in the ng/ml range and showed no correlation with disease severity when measured by either extension score or clinical score. However, the difference in neopterin levels between these two blood collection sites did correlate with atherosclerotic severity when measured by the Gensini score. Analysis by this means may therefore have permitted the detection of smaller concentration changes than the analysis of peripheral blood, as described for instance, by Schumacher *et al.* (1997). Nevertheless, it still failed to determine the actual concentration of neopterin within the local inflammatory environment of the plaque. This study also did not attempt to address the question of pterin and antioxidant activity. The current study has therefore investigated these issues by measuring total neopterin (neopterin plus 7,8-NP) in two inflammatory environments – pus removed from various sites of acute inflammation and plaque removed from the carotid artery. In an effort to identify any potential antioxidant activity of pterin, markers of oxidation were also monitored in each sample and analyzed for the existence of correlations with neopterin.

6.2 Results

6.2.1 Pus

The nineteen patients, eleven women and eight men, who consented to participate in this study had a mean age of 57.8 ± 4.0 years, and a median of 61 years (Table 6.1). All presented with symptoms of acute inflammation, though the exact location varied from one person to the next. Data regarding each patient's medical history and prescriptions was limited. Nevertheless, the majority did not appear diabetic, with only the patients numbered 7 and 10 being listed as possibly diabetic while 11 and 17 were described as mildly diabetic (data not shown). It was also known that most patients were prescribed antibiotics prior to the removal of pus in an attempt to control any microbial growth at the site of inflammation.

Table 6.1 General characteristics of patients with acute inflammation.

Sample Number	Sex	Age	Location of acute inflammation
1	Female	89	Gall bladder
2	Female	69	Anterior abdominal wall
3	Male	48	Pararectal, lower pelvic region
4	Male	73	Paracoli, lower pelvic region
5	Male	68	Right hand side flank
6	Female	41	Right hand side anterior plural space
7	Female	24	Fistula/abscess right hand side
8	Female	43	Gallbladder fossa
9	Male	61	Peripancreatic
10	Female	49	Paracoli region
11	Male	51	Gallbladder fossa
12	Male	44	Right hand side plural space
13	Female	64	Pelvis
14	Male	89	Abdominal cavity
15	Male	53	Lower abdomen
16	Female	62	Subphrenic
17	Female	74	Pelvis
18	Female	34	Left pelvis
19	Female	62	Subphrenic

6.2.1.1 Neopterin in Pus

Neopterin (including oxidized 7,8-NP) in the 19 samples of pus was found to range between 0.05μM and 1.28μM, with lower and upper quartile concentrations of 0.30μM and 0.69μM, respectively (Table 6.2). While individual neopterin levels for each patient are listed in Appendix I, a combined analysis of all patients produced a mean of 0.51 ± 0.07μM neopterin (Table 6.2).

Classifying subjects by gender gave an average neopterin concentration of 0.54 ± 0.07μM for females but 0.45 ± 0.14μM for males at these sites of acute inflammation (Figure 6.1). However, the slightly lower neopterin content associated with males was not statistically significant. Age also did not appear to be a major determinant of neopterin concentrations in the pus. A comparison of these two parameters yielded no significant correlation (Figure 6.2).

Table 6.2 Statistics for markers of oxidation and inflammation in pus.
The 19 pus samples were analyzed for PB-DOPA, dityrosine, carbonyls, TBARS, vitamin E and neopterin (including oxidized 7,8-NP). Averages and medians, as well as lower and upper quartiles, were subsequently calculated for each parameter. See Appendix I for a table showing concentration data for each patient.

Parameter	Mean ± SEM	Median (Range)	Lower & Upper Quartile
PB-DOPA (μM)	15.24 ± 4.99	7.22 (1.86 - 96.9)	4.16, 19.27
Dityrosine (μM)	1.84 ± 1.06	0.20 (0.00 - 19.28)	0.09, 0.57
Carbonyls (μM)	197.4 ± 37.53	164.7 (5.79 - 471.8)	44.07, 359.6
TBARS (μM)	3.29 ± 0.54	2.48 (0.81 – 10.06)	1.22, 5.27
Vitamin E (μM)	23.07 ± 3.04	21.89 (2.96 – 46.49)	11.27, 35.15
Neopterin (μM)	0.51 ± 0.07	0.52 (0.05 – 1.28)	0.30, 0.69

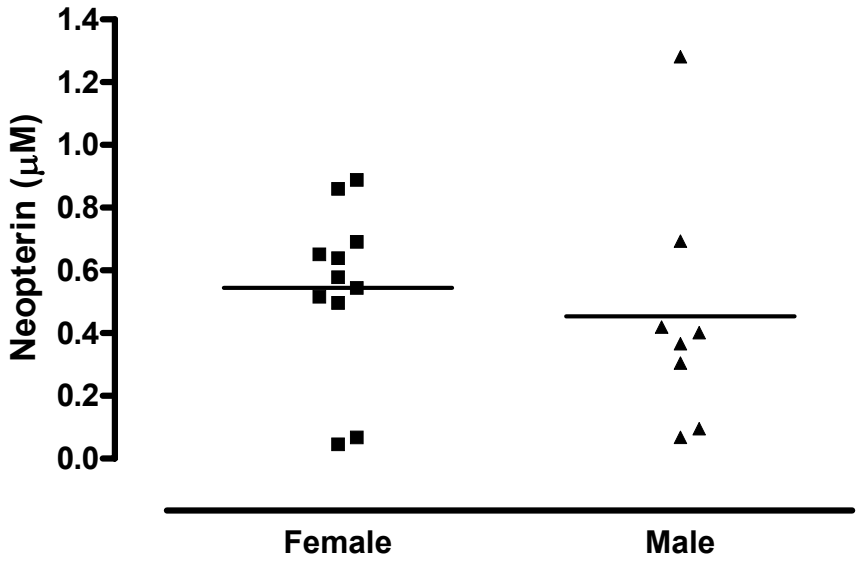


Figure 6.1 Effect of sex on neopterin levels at sites of acute inflammation.
Pus was removed from eleven female patients and eight male patients at sites of acute inflammation and measured for neopterin (including oxidized 7,8-NP) via HPLC.

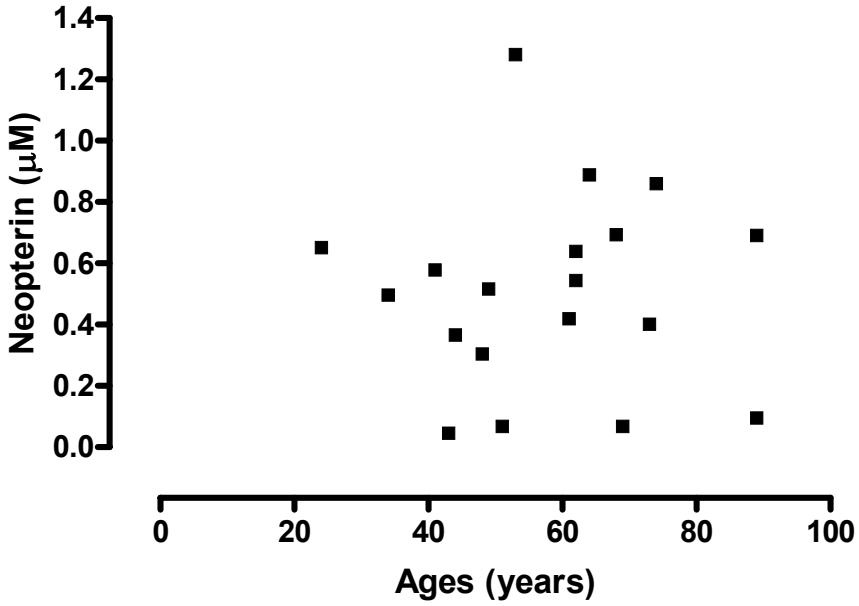


Figure 6.2 Effect of age on neopterin levels at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for neopterin (including oxidized 7,8-NP) via HPLC. Neopterin was subsequently compared against the age of each corresponding patient.

Due to the clinical environment and the health of the patients, it was not possible to obtain blood samples for research purposes. However, Christchurch Hospital was able to provide data, for 14 of the 19 patients, describing macrophage and white cell numbers in the blood. These cells and neopterin are both generally regarded as markers of

immune activation. Despite this, no significant correlation was observed between neopterin in the pus and macrophage cell counts in the blood (Figure 6.3a). Pus neopterin did appear to increase as the concentration of white blood cells increased but, with r^2=0.20 and p=0.096, this trend also failed to reach significance (Figure 6.3b).

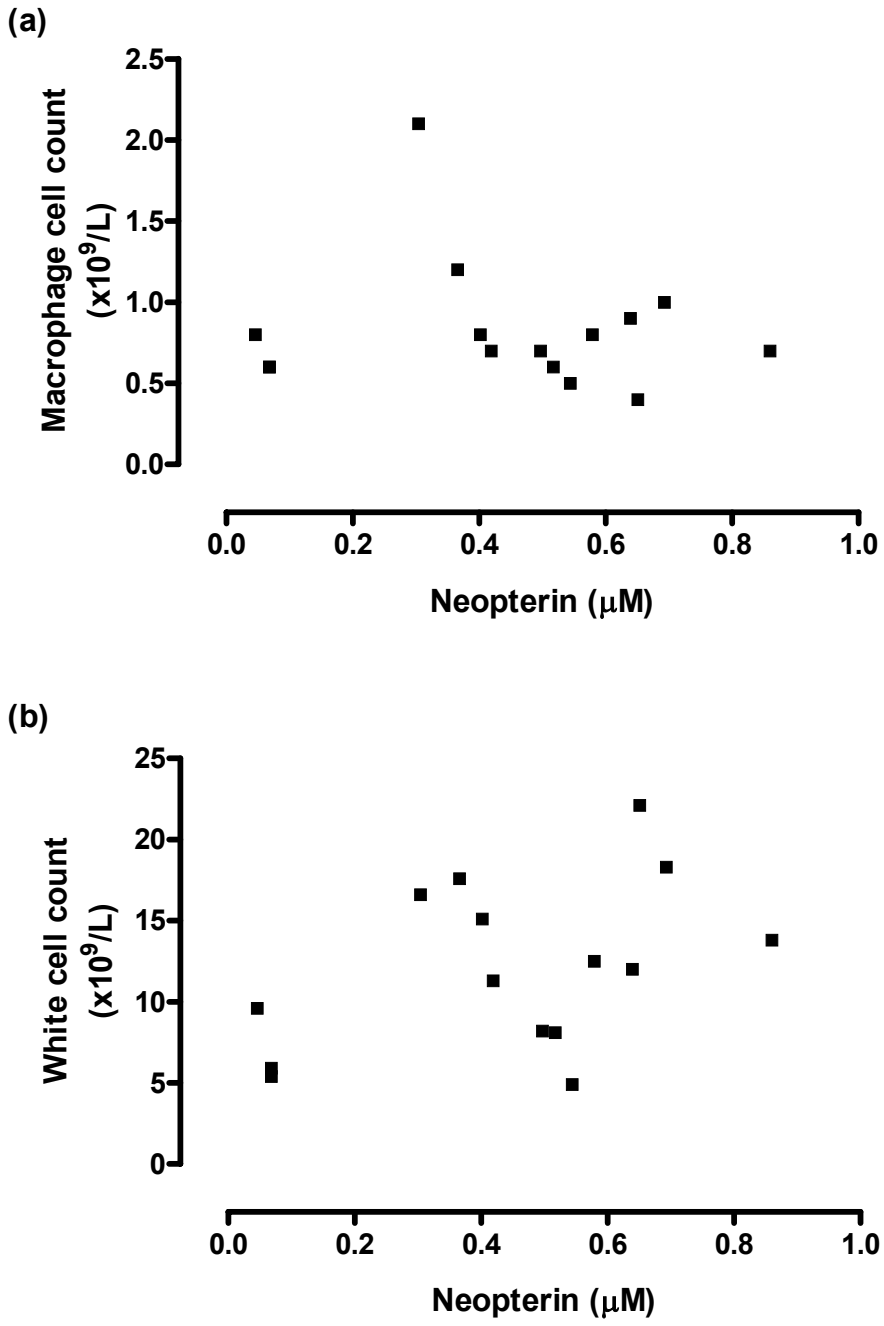


Figure 6.3 Relationship between blood cell count and neopterin at sites of acute inflammation.

Pus was removed from 19 patients at sites of acute inflammation and measured for neopterin (including oxidized 7,8-NP) via HPLC. Blood screening by Christchurch Hospital provided counts of (a) macrophage numbers and (b) white cell numbers.

If 7,8-NP were to exhibit antioxidant activity at sites of inflammation, it is possible that correlations between its oxidation product (neopterin) and various markers of oxidative attack would be detectable in the pus. Hydroperoxides were not analyzed due to their lack of stability over prolonged periods (Fu *et al.*, 1998) but several other protein oxidation products, including PB-DOPA (Figure 6.4), dityrosine (Figure 6.5) and carbonyls (Figure 6.6), were able to be monitored. Despite comparing neopterin concentrations against all three of these oxidized protein components, no relationship was detected. Likewise, the protein concentration in this inflammatory environment remained independent of the pus neopterin content (Figure 6.7).

Patient 2 possessed the highest concentrations of PB-DOPA, dityrosine and protein. However, even removing this outlier from the above analyses did not produce a significant association between neopterin and oxidized protein in pus (data not shown).

This lack of a correlation with neopterin also extended to TBARS, a lipid oxidation product (Figure 6.8). A comparison of TBARS and neopterin levels in the inflammatory material yielded a highly insignificant $p=0.58$ and $r^2=0.018$.

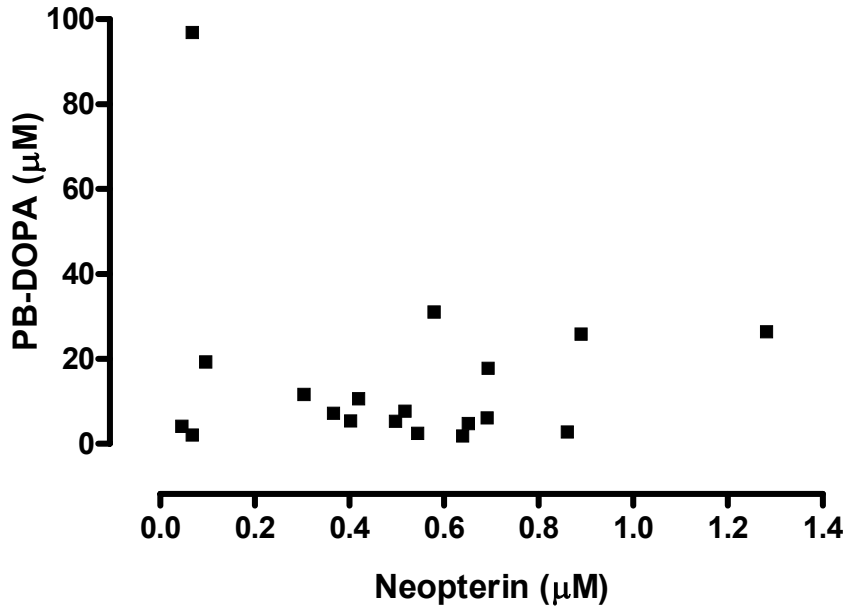


Figure 6.4 Relationship between total neopterin and PB-DOPA at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for neopterin (including oxidized 7,8-NP) and PB-DOPA via HPLC.

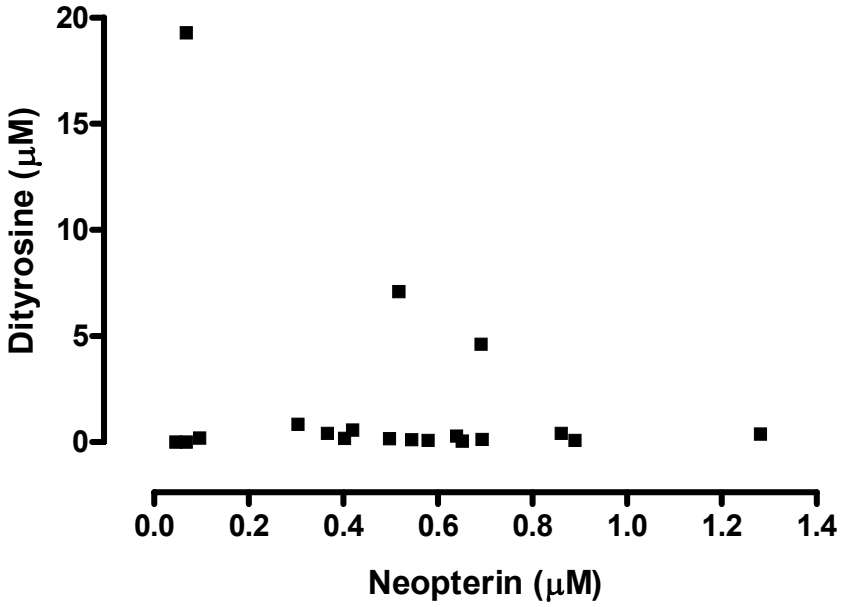


Figure 6.5 Relationship between total neopterin and dityrosine at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for neopterin (including oxidized 7,8-NP) and dityrosine via HPLC.

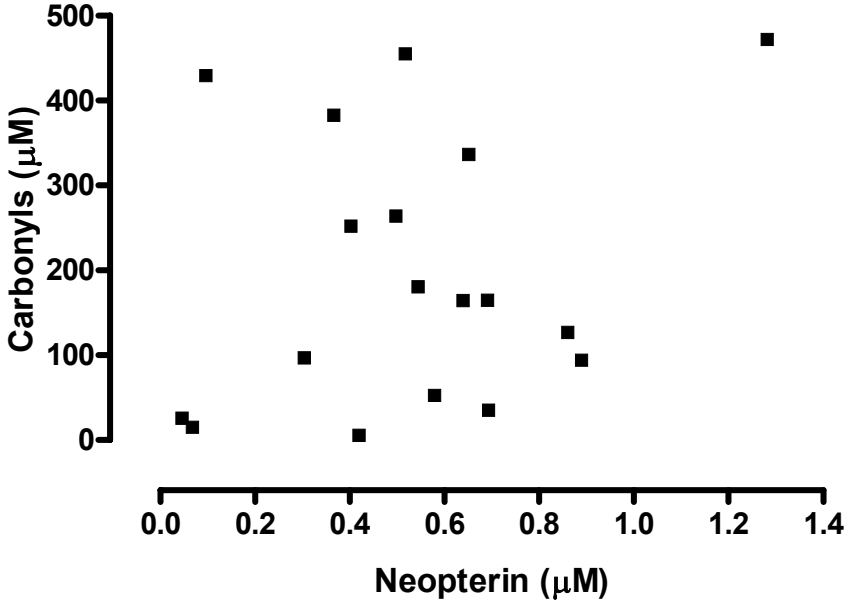


Figure 6.6 Relationship between total neopterin and carbonyls at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for neopterin (including oxidized 7,8-NP) and protein carbonyls via HPLC and spectrophotometer, respectively.

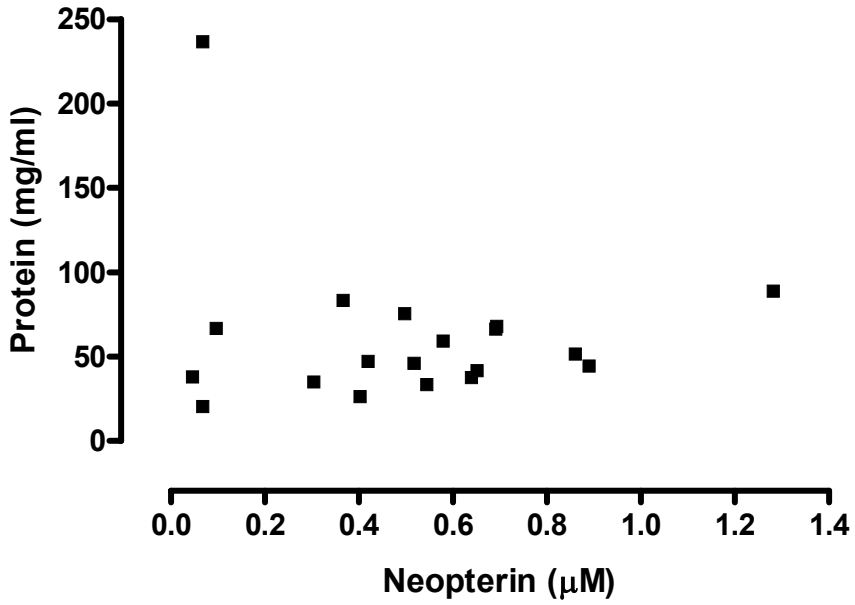


Figure 6.7 Relationship between total neopterin and protein at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for neopterin (including oxidized 7,8-NP) and protein via HPLC and spectrophotometer.

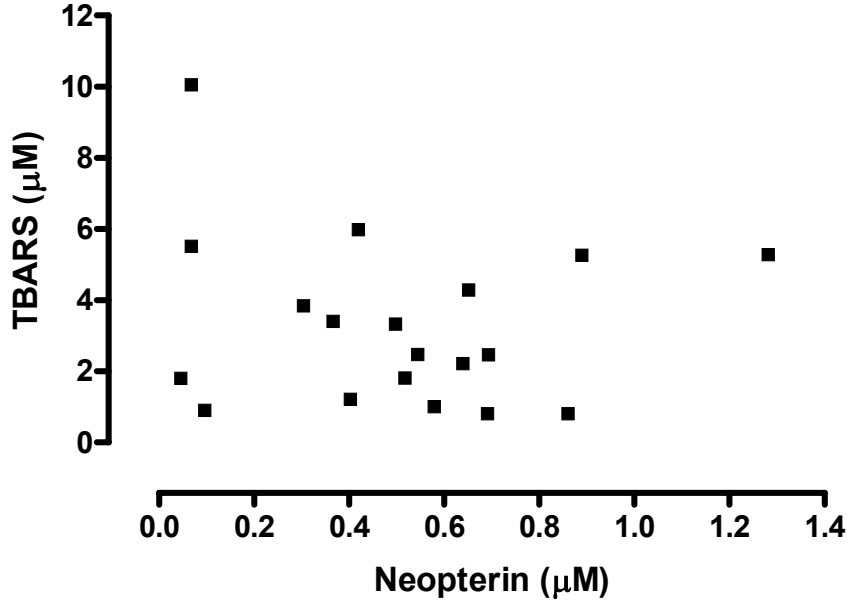


Figure 6.8 Relationship between neopterin and lipid peroxidation at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for neopterin (including oxidized 7,8-NP) and TBARS via HPLC.

By contrast, a trend between vitamin E and neopterin was identifiable. An increase in the pus content of one parameter tended to be associated with a rise in the pus content of the other (Figure 6.9a). However, this positive relationship only reached significance ($p<0.05$, $r^2=0.228$) when expressing both vitamin E and neopterin per gram of pus protein (Figure 6.9b).

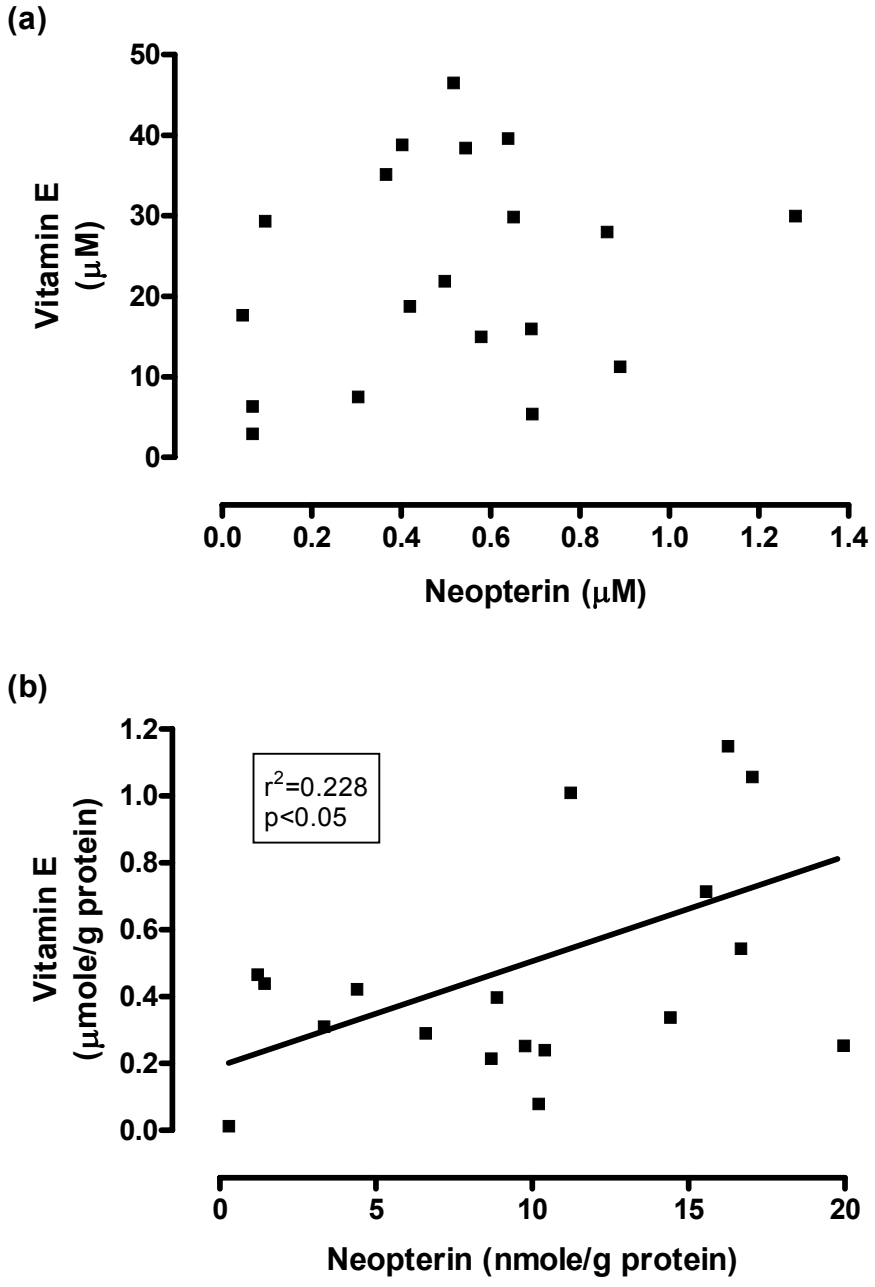


Figure 6.9 Relationship between neopterin and vitamin E at sites of acute inflammation.

Pus was removed from 19 patients at sites of acute inflammation and measured for neopterin (including oxidized 7,8-NP) and vitamin E via HPLC. Data is expressed in (a) micromoles per litre or (b) micro/nano-moles per gram of protein. A trend line is included to illustrate the correlation.

It should also be noted that vitamin E in the pus was associated with a mean concentration of 23.07 ± 3.04μM (Table 6.2). This represents a vitamin E level approximately 45 times larger than the corresponding neopterin average of 0.51 ± 0.07μM. A similar concentration disparity was noted when expressing vitamin E and neopterin in moles per gram of protein or when comparing their median values.

6.2.1.2 Comparisons Between Markers of Oxidation in Pus

In addition to studying the relationships of the various oxidation markers with neopterin, it was also possible to investigate associations between the markers themselves. A direct comparison of median values for the three protein oxidation products revealed that, with a median concentration of 164.7μM and an interquartile range between 44.07-359.6μM, carbonyls were present at the highest concentration in pus (Table 6.2). PB-DOPA, by comparison, was approximately 23-fold lower and was associated with a median and interquartile range of only 7.22μM and 4.16-19.27μM, respectively. Dityrosine concentrations were generally even smaller, as shown by the median of only 0.20μM and an interquartile range of 0.09-0.57μM.

No difference between any of the oxidation parameters and age or gender was identified (data not shown), but nine other correlations were detected, while a tenth verged on significance. In the case of the latter, the pus protein content (in mg/ml) showed a tendency to increase as the micromolar quantity of carbonyls increased. This association produced a p value of exactly 0.05 and an r^2 Pearson value of 0.22 (data not shown). The positive relationship between protein and markers of oxidation was more significant for micromolar quantities of PB-DOPA (Figure 6.10), dityrosine (Figure 6.11) and even TBARS (Figure 6.12). These correlations were strongest for the products of protein oxidation, with $p<0.001$ for both PB-DOPA and dityrosine, and an r^2 of 0.821 and 0.718, respectively. In comparison, TBARS and protein concentrations at the site of acute inflammation were significantly associated only by $p<0.01$ and $r^2=0.378$. Patient 2 had a strong effect on the above correlations, and significance was lost if this patient's data was removed from analysis (data not shown).

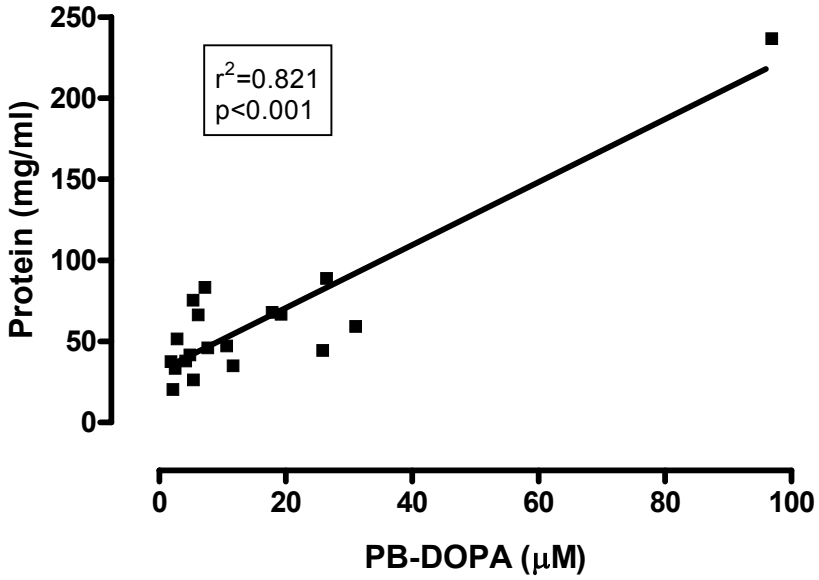


Figure 6.10 Correlation between PB-DOPA and protein at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for PB-DOPA and protein via HPLC and spectrophotometer, respectively. A trend line is included to illustrate the correlation.

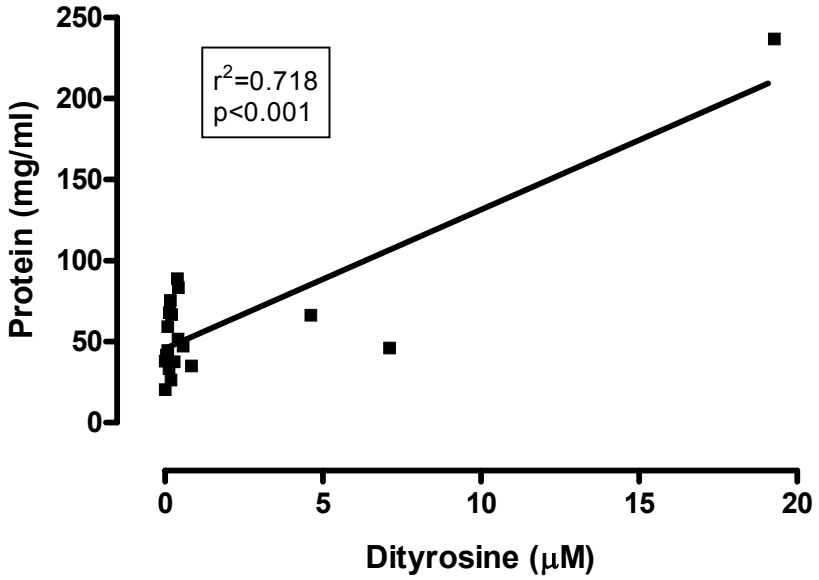


Figure 6.11 Correlation between dityrosine and protein at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for dityrosine and protein via HPLC and spectrophotometer, respectively. A trend line is included to illustrate the correlation.

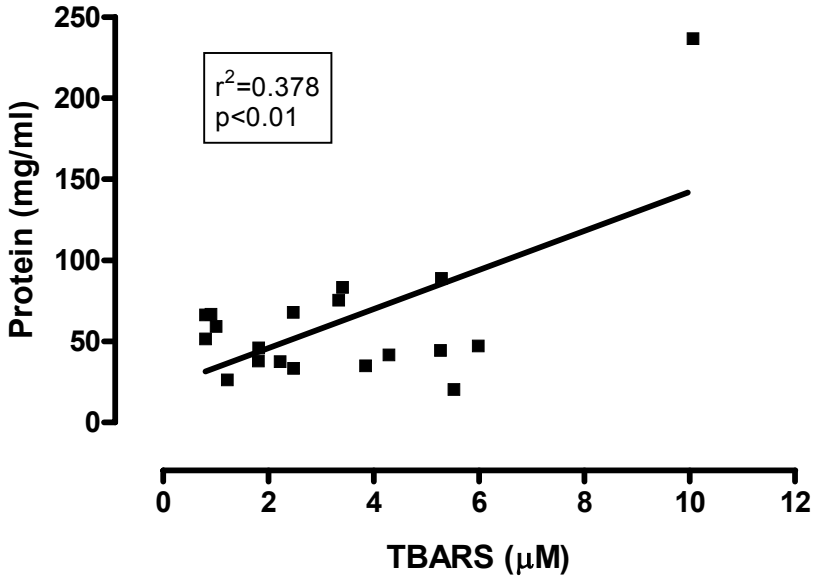


Figure 6.12 Correlation between TBARS and protein at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for TBARS and protein via HPLC and spectrophotometer, respectively. A trend line is included to illustrate the correlation.

A significant correlation was also apparent between PB-DOPA and dityrosine, the two tyrosine oxidation products. With a $p<0.001$ and $r^2=0.66$, the increase in pus-associated dityrosine was determined to be proportional to the increase in pus-associated PB-DOPA (Figure 6.13).

Each of the tyrosine oxidation products were, likewise, positively associated with the lipid peroxidation marker, TBARS. The relationship between PB-DOPA and TBARS was strongest, as verified by $p<0.01$ and $r^2=0.443$ (Figure 6.14). Nevertheless, a statistical comparison of dityrosine and TBARS revealed an association that was also significant, with $p<0.05$ and $r^2=0.307$ (Figure 6.15).

It should be noted, however, that the three correlations between PB-DOPA, dityrosine and TBARS all lost significance if the outlier from patient 2, corresponding to 96.90μM PB-DOPA, 19.28μM dityrosine and 10.06μM TBARS, was removed from analysis (data not shown). Significance was also lost if the data was compared on a mole per gram of protein basis (data not shown).

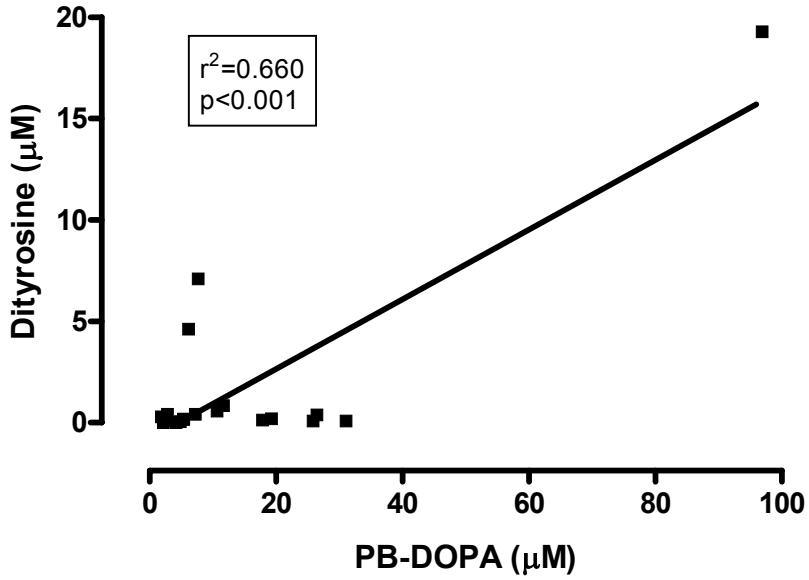


Figure 6.13 Correlation between PB-DOPA and dityrosine at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for PB-DOPA and dityrosine via HPLC. A trend line is included to illustrate the correlation.

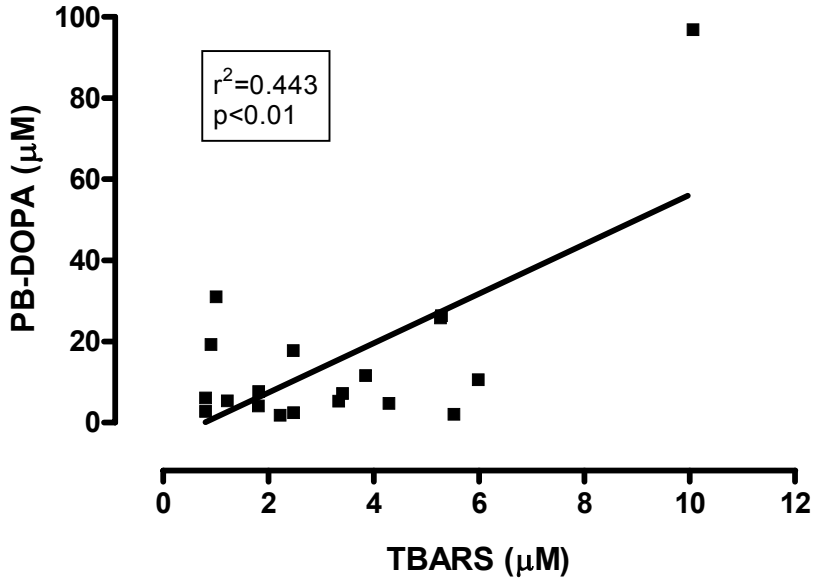


Figure 6.14 Correlation between TBARS and PB-DOPA at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for TBARS and PB-DOPA via HPLC. A trend line is included to illustrate the correlation.

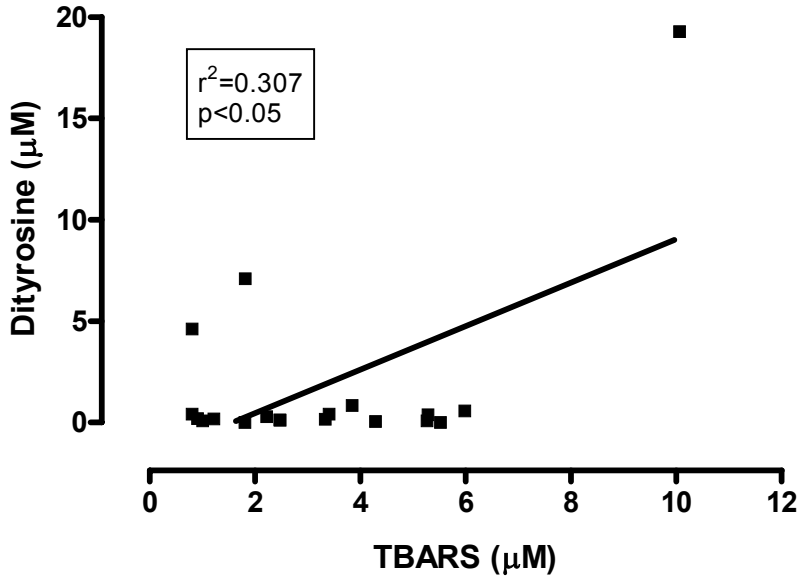


Figure 6.15 Correlation between TBARS and dityrosine at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for TBARS and dityrosine via HPLC. A trend line is included to illustrate the correlation.

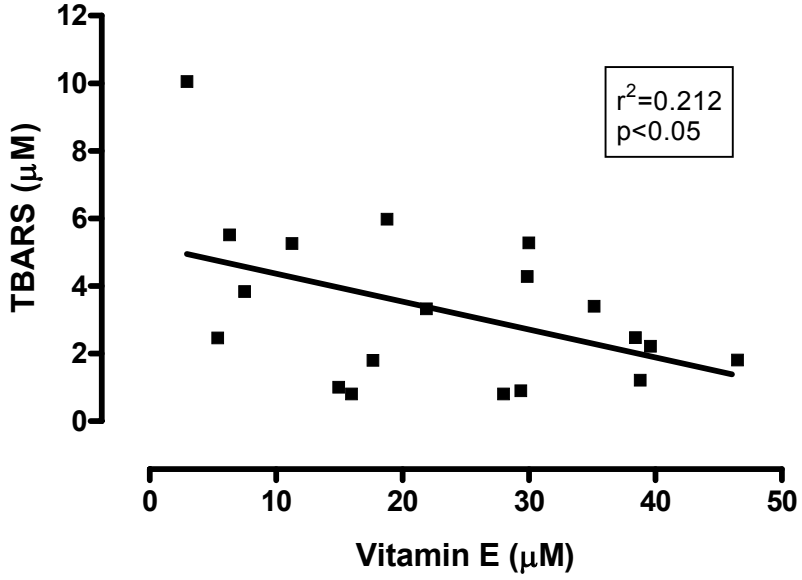


Figure 6.16 Correlation between TBARS and vitamin E at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for TBARS and vitamin E via HPLC. A trend line is included to illustrate the correlation.

In contrast to the lack of association with neopterin, a couple of oxidation markers were shown to correlate negatively with vitamin E. In particular, the TBARS (Figure 6.16) and PB-DOPA (Figure 6.17a) concentrations both decreased as the quantity of

vitamin E increased. Statistical analysis gave $p< 0.05$ and $r^2=0.212$ for each correlation. Once again, the relationship lost significance if the data for patient 2 was excluded from analysis (data not shown). The correlation between PB-DOPA and vitamin E could be regained, even in the presence of patient 2, after normalizing PB-DOPA against the protein content of pus ($p<0.05$, $r^2=0.230$; Figure 6.17b).

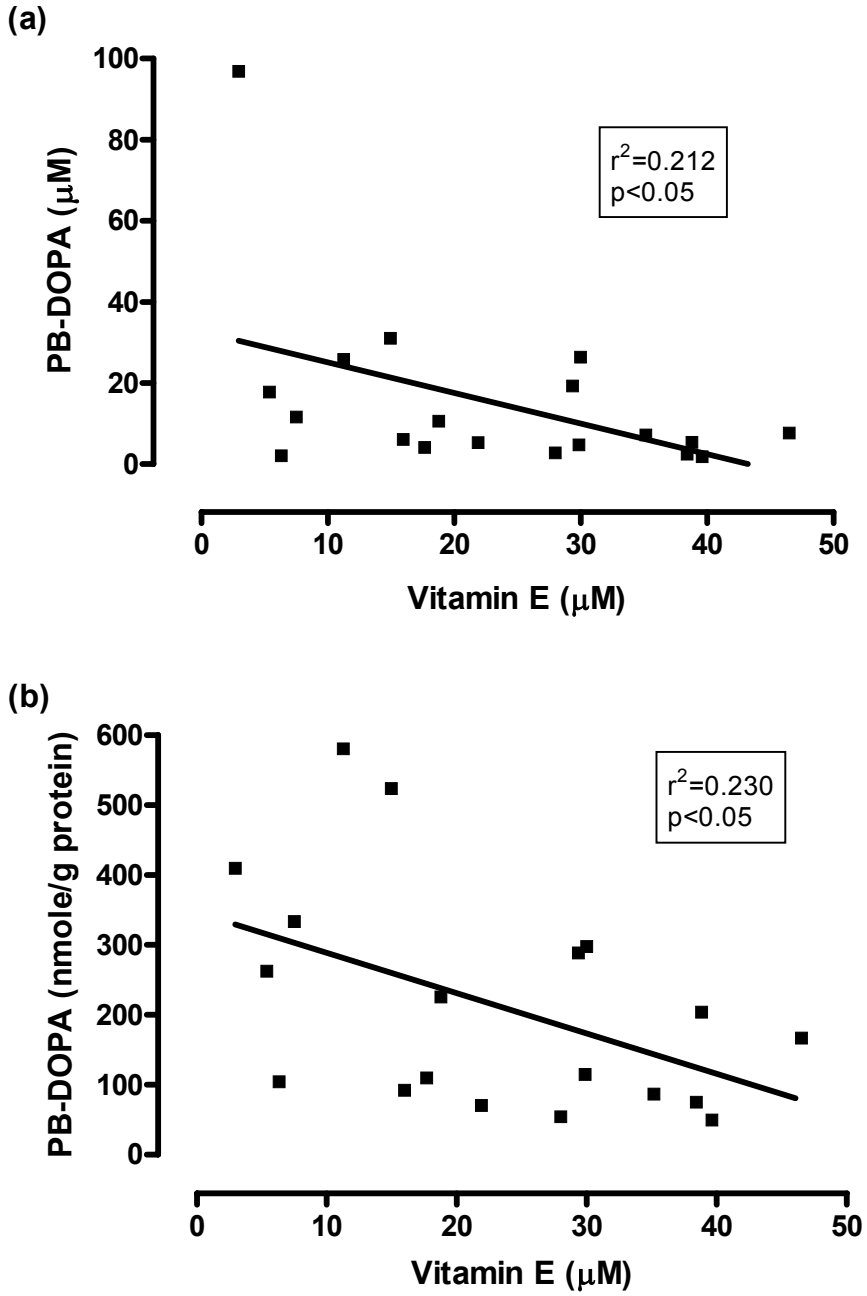


Figure 6.17 Correlation between PB-DOPA and vitamin E at sites of acute inflammation.

Pus was removed from 19 patients at sites of acute inflammation and measured for PB-DOPA and vitamin E via HPLC. Vitamin E is expressed in micromoles per litre while PB-DOPA is expressed in (a) micromoles per litre or (b) nanomoles per gram of protein. A trend line is included to illustrate the correlation.

Vitamin E could also be correlated to the carbonyl level in the inflammatory material, with statistical analysis producing a $p<0.01$ and $r^2=0.492$ (Figure 6.18). This association was unexpectedly observed to be positive, and it remained so, even when data was expressed in moles per gram of protein (data not shown).

It should be noted that patient two was not included in the carbonyl analysis. When spectrophotometrically detecting carbonyl in the pus from this patient, values smaller than the baseline absorbance were consistently recorded.

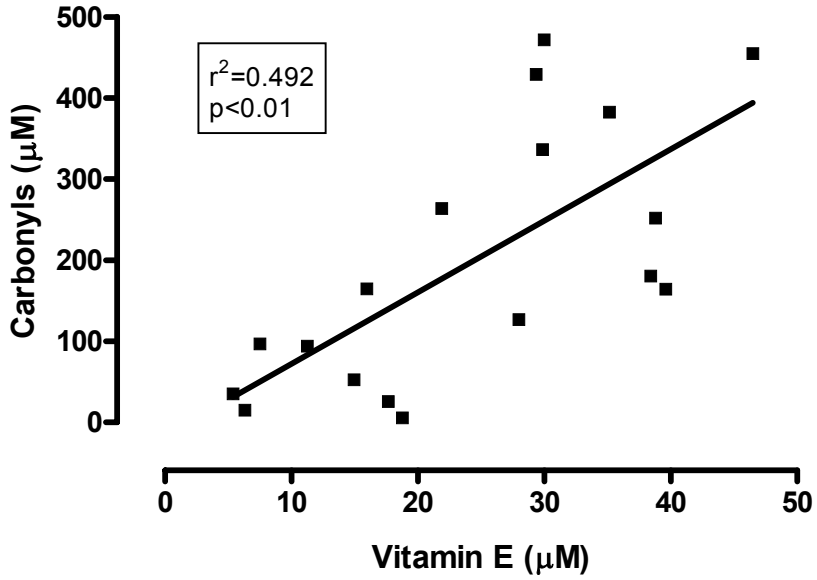


Figure 6.18 Correlation between vitamin E and carbonyls at sites of acute inflammation.
Pus was removed from 19 patients at sites of acute inflammation and measured for vitamin E and protein carbonyls via HPLC and spectrophotometer, respectively. A trend line is included to illustrate the correlation.

6.2.2 Plaque

Plaque was removed from the carotid artery of two patients as part of a pilot study aiming to monitor neopterin levels throughout this chronic inflammatory environment. Each plaque was therefore subsequently divided into six or seven sections, with each piece being analyzed for the presence of neopterin and a range of oxidation markers.

The two patients enrolled in this pilot study were both men in their early eighties (Table 6.3). Neither was diabetic, both were ex-smokers, but only patient 2 was listed as hypertensive. The reported degree of stenosis also differed between the two patients, with the first scoring 80% and the second 60-69% when judged by ultrasound. Conversely, an inspection of the cross-sections photographed after plaque dissection suggested that the carotid artery from patient 1 generally contained less gruel than patient 2 (Figure 6.19 & Figure 6.29). Only the last section, number six, appeared significantly stenosed in patient 1 while all seven sections of patient 2 were filled with gruel.

As expected for heart patients, both were prescribed aspirin (Table 6.3). The first was also prescribed a drug to treat ulcers and/or gastroesophageal reflux (Omeprazole) while the second was prescribed a loop diuretic drug (Frusemide), a hypertensive drug (Losartan) and an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Atorvastatin).

Table 6.3 Characteristics of two carotid endarterectomy patients.

	Patient 1	**Patient 2**
Sex	Male	Male
Age (years)	80	83
Smoking Habit	Ex-smoker	Ex-smoker
Hypertension	No	Yes
Diabetic	No	No
Extent of stenosis	80%	60-69%
Symptoms	Stroke, Amaurosis fugax	Amaurosis fugax
Medication	Aspirin, Omeprazole	Aspirin, Atorvastatin, Frusemide, Losartan

6.2.2.1 Trends in the Carotid Plaque from Patient 1

Approximately 28mm of plaque was removed from the right internal carotid artery of patient 1 (Figure 6.19). The subsequent dissection created six sections, each representing 4-5mm of the plaque length and 0.05-0.328g of plaque weight.

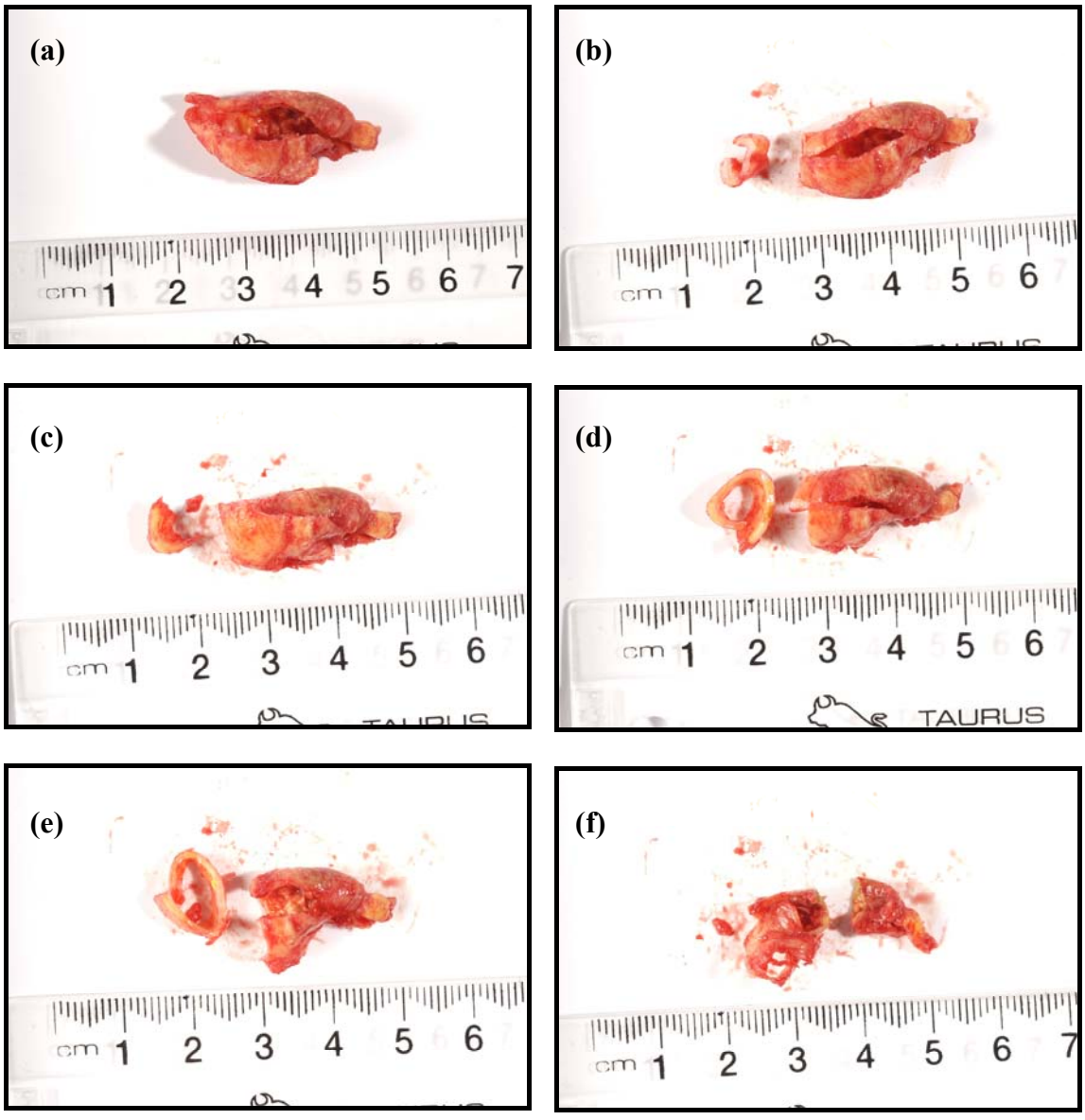


Figure 6.19 Cross-sections of the internal carotid artery from patient 1.
The (a) right internal carotid artery was removed from patient 1 and dissected into six segments, labelled (b) section one, (c) section two, (d) section three, (e) section four, (f) section five (left) and section six (right). This latter section represents the site proximal to the bifurcation.

i) Neopterin

When calculated across all sections, neopterin (including oxidized 7,8-NP) in plaque 1 averaged a concentration of 0.19 ± 0.10 nmole/g plaque (Figure 6.20). However, with five of the six sections ranging only between 0.06 and 0.12 nmole/g plaque, the median was restricted to 0.09 nmole/g plaque. This does not detract from the existence of a spike in section four, corresponding to a neopterin content of 0.71nmole/g plaque. Given a plaque density of approximately 1g/ml, this equates to a peak molar concentration of 0.71μM neopterin.

Three examples of HPLC neopterin traces for plaque 1 are displayed in Figure 6.21. As the standard eluted at 5.158 minutes (data not shown), peak number four corresponds to the neopterin in sections three (Figure 6.21a) and four (Figure 6.21b) while peak number three corresponds to the neopterin in section five (Figure 6.21c). These traces further highlight the large neopterin peak detected in section four compared to the significantly lower quantity of neopterin in the surrounding sections.

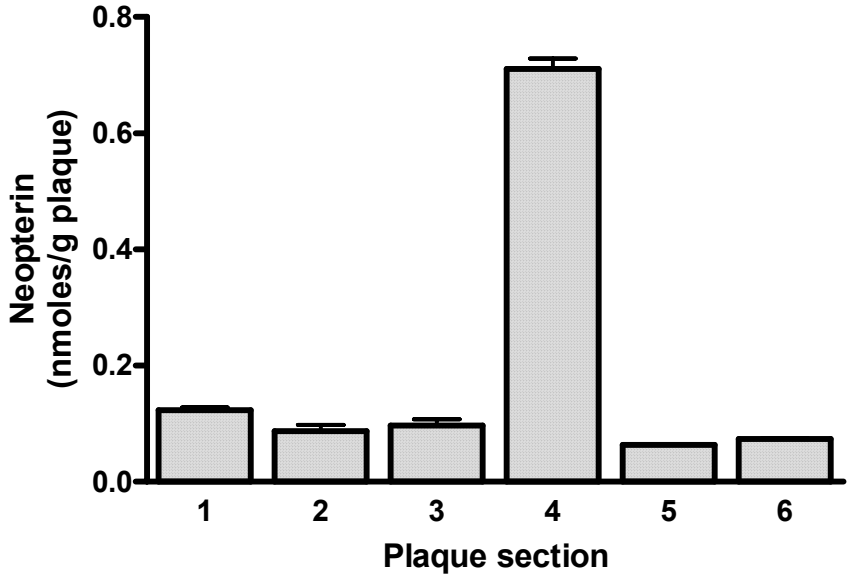
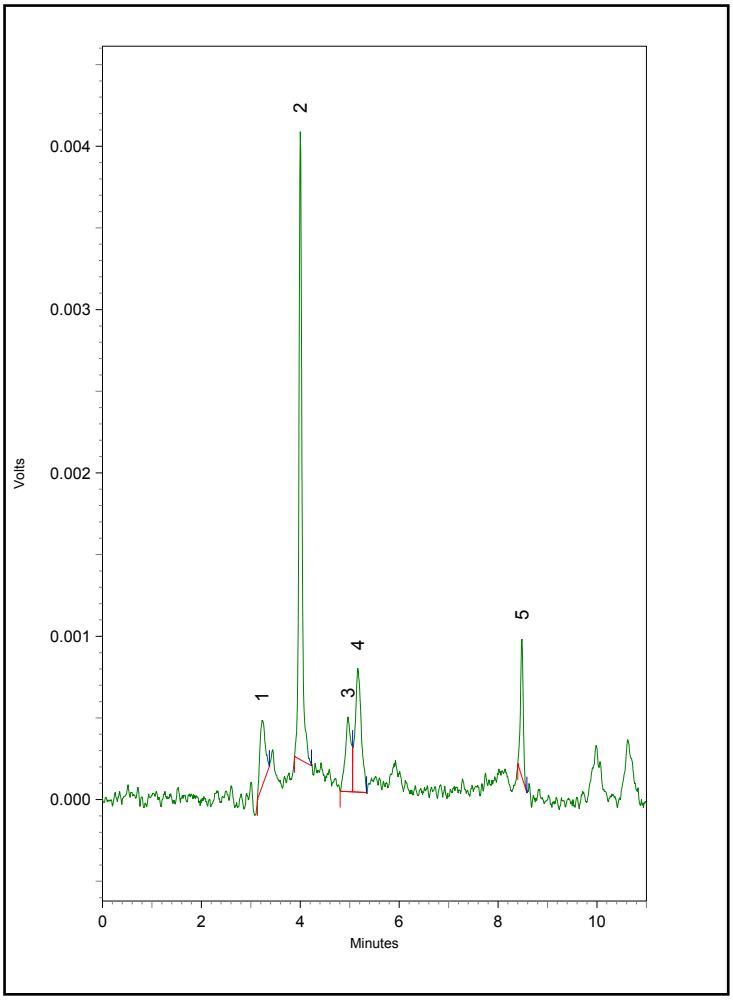
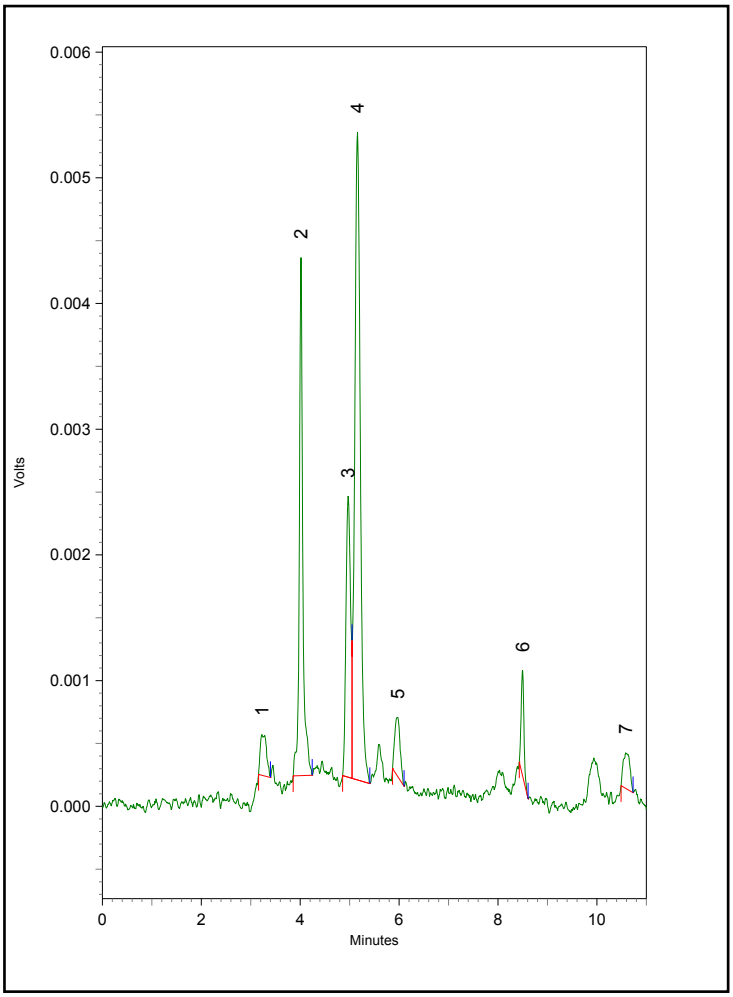


Figure 6.20 Concentration of neopterin in six sections of plaque 1.
After dissecting the right internal carotid artery into six sections, each piece was analyzed by HPLC for neopterin (including oxidized 7,8-NP). Results are displayed as mean ± SEM of triplicates.

(a)

Peak #	Retention Time	Area
1	3.225	3024
2	4.000	16525
3	4.967	3702
4	**5.167**	**6588**
5	8.483	3330





(b)

Peak #	Retention Time	Area
1	3.225	2845
2	4.017	20245
3	4.967	14568
4	**5.158**	**40588**
5	5.975	3551
6	8.500	3312
7	10.583	2689

(c)

Peak #	Retention Time	Area
1	4.017	33671
2	4.983	6995
3	**5.150**	**7706**
4	8.467	4431
5	10.533	2980

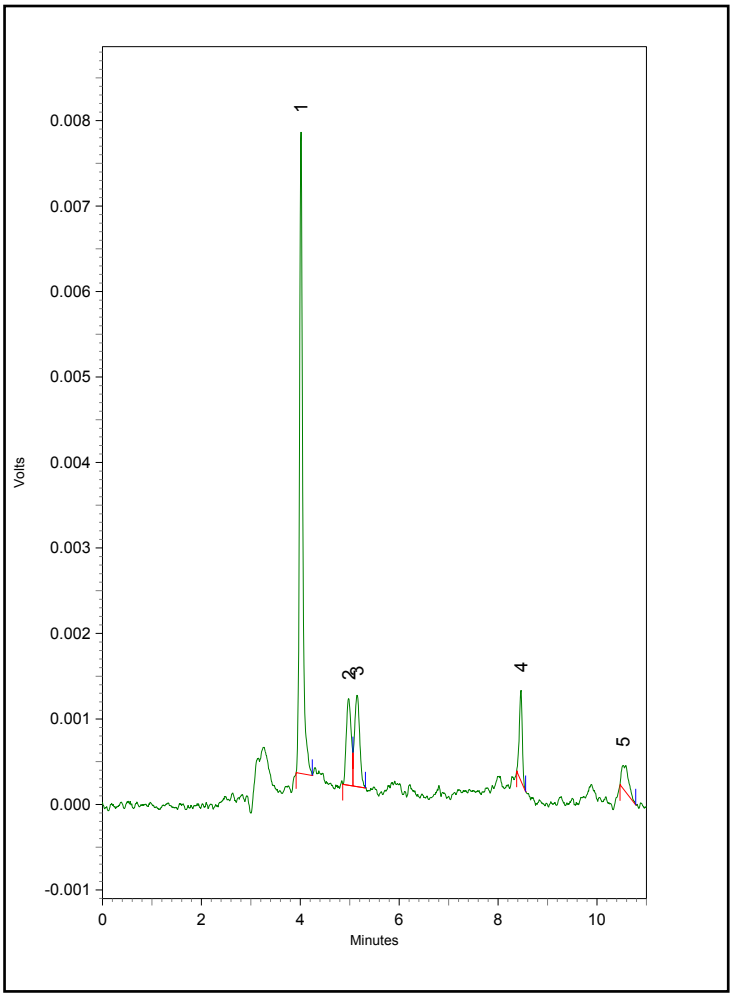


Figure 6.21 HPLC neopterin traces representative of three sections in plaque 1. Homogenized plaque was incubated with TCA and acidic iodine. Neopterin (including oxidized 7,8-NP) was subsequently detected via HPLC, using excitation and emission wavelengths of 353nm and 438nm, respectively, and a mobile phase of 20mM ammonium phosphate (pH 6) with 5% methanol. HPLC neopterin traces are presented for (a) section three, (b) section four and (c) section five of plaque 1. The peaks corresponding to neopterin occur at (a) peak four, (b) peak four and (c) peak three.

ii) Protein and Cholesterol

Protein concentrations per gram of plaque did not vary significantly throughout the first five sections of plaque 1 (Figure 6.22). Section six, however, was associated with a significant ($p<0.001$) increase to 117 ± 2.56 mg/g plaque. This represented a jump of more than 50% when compared to the protein content in the preceding plaque section.

Cholesterol levels, by contrast, showed a gradual rise that finally reached significance ($p<0.01$) from section one by section four (Figure 6.23). This was followed by a dramatic 2.3-fold increase in the cholesterol content, at section five, to 76.13 ± 2.68 µmole/g plaque ($p<0.001$). A subsequent, and significant, drop to 49.61 ± 2.15 µmole/g

plaque ($p<0.001$) nevertheless remained elevated compared to the first four sections ($p<0.001$ against sections one to three but $p<0.01$ against section four).

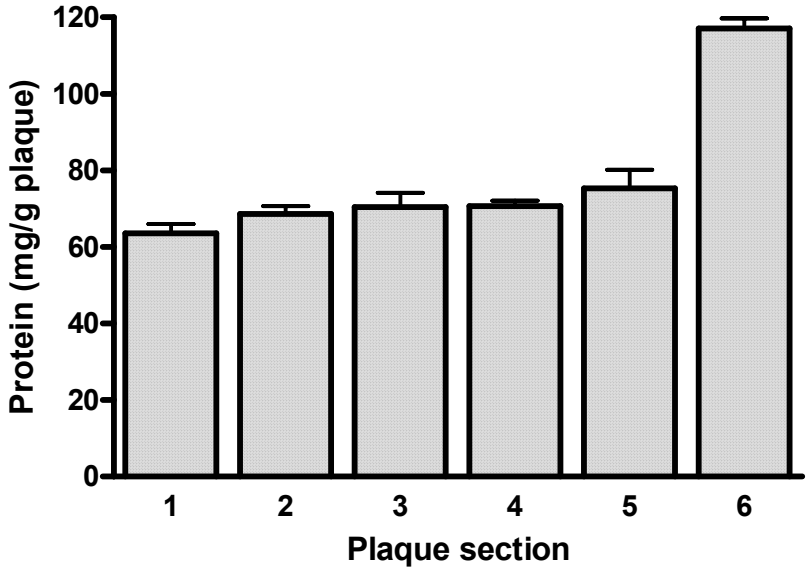


Figure 6.22 Concentration of protein in six sections of plaque 1.
After dissecting the right internal carotid artery into six sections, each piece was analyzed by the BCA protein determination kit for protein. Results are displayed as mean ± SEM of triplicates.

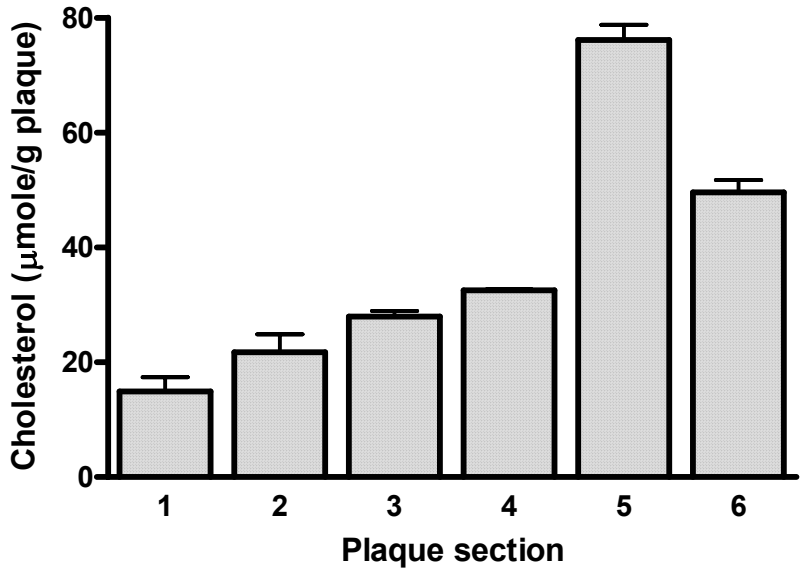


Figure 6.23 Concentration of cholesterol in six sections of plaque 1.
After dissecting the right internal carotid artery into six sections, each piece was analyzed by spectrophotometer for cholesterol. Results are displayed as mean ± SEM of triplicates.

iii) Markers of Oxidation

PB-DOPA levels throughout the six sections of plaque 1 displayed no obvious trend, and this remained the case whether expressing protein oxidation in nmoles per gram of plaque (Figure 6.24) or nmoles per gram of protein (data not shown). In fact, the 13.48 ± 1.63nmole/g plaque concentration recorded for section one differed significantly only from the PB-DOPA content of section two ($p<0.05$).

Excluding section two, all other segments of plaque 1 were found to contain detectable quantities of dityrosine (Figure 6.25). Dityrosine concentrations were, however, consistently lower than the level of PB-DOPA associated with each respective section of plaque (Figure 6.24 & Figure 6.25). In further contrast to PB-DOPA, a dityrosine trend was noted. Levels began to increase significantly in the final two segments ($p<0.001$), peaking at 1.29 ± 0.03 nmoles of dityrosine per gram of plaque in section six.

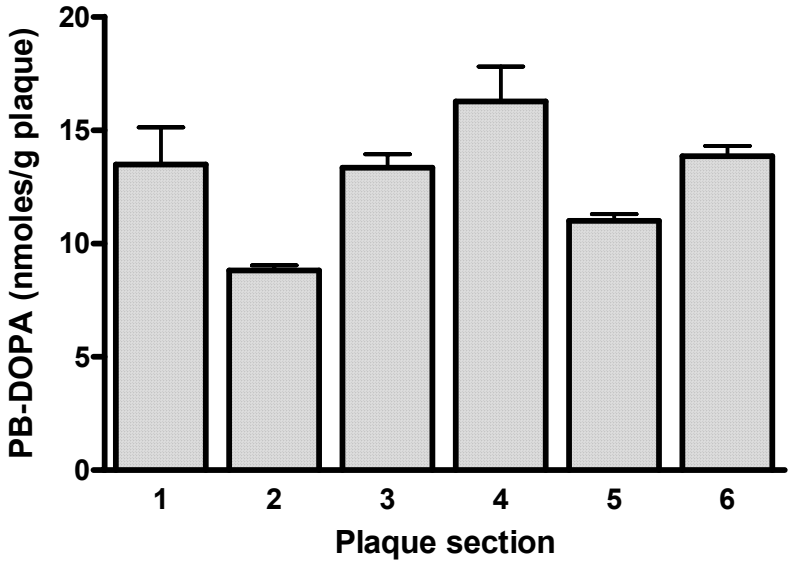


Figure 6.24 Concentration of PB-DOPA in six sections of plaque 1.
After dissecting the right internal carotid artery into six sections, each piece was analyzed by HPLC for PB-DOPA. Results are displayed as mean ± SEM of triplicates.

Carbonyls, like dityrosine, did not represent a significant component of section 2 (Figure 6.26). Excluding this segment, carbonyls showed a general tendency to increase along the length of the plaque. A significant elevation above the first segment was therefore finally attained in section five, at the maximum concentration of 301.8 ± 23.15 nmole/g plaque ($p<0.05$). The subsequent, dramatic decrease ($p<0.001$) observed for section six resulted in a carbonyl content that was lower, but not significantly

different, from the section one carbonyl concentration. Despite this drop, carbonyls remained more numerous than the PB-DOPA in each of the six sections analyzed (Figure 6.24 & Figure 6.26). This difference lacked significance only for the second segment of plaque 1.

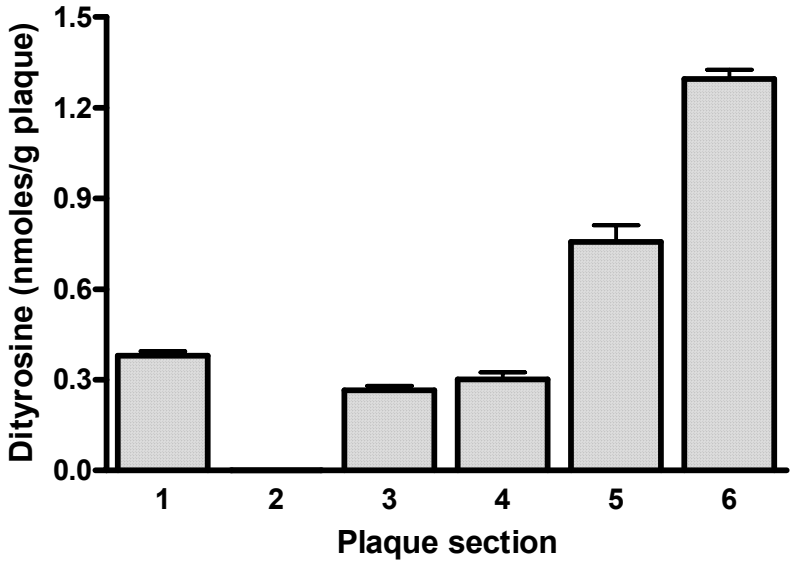


Figure 6.25 Concentration of dityrosine in six sections of plaque 1.
After dissecting the right internal carotid artery into six sections, each piece was analyzed by HPLC for dityrosine. Results are displayed as mean ± SEM of triplicates.

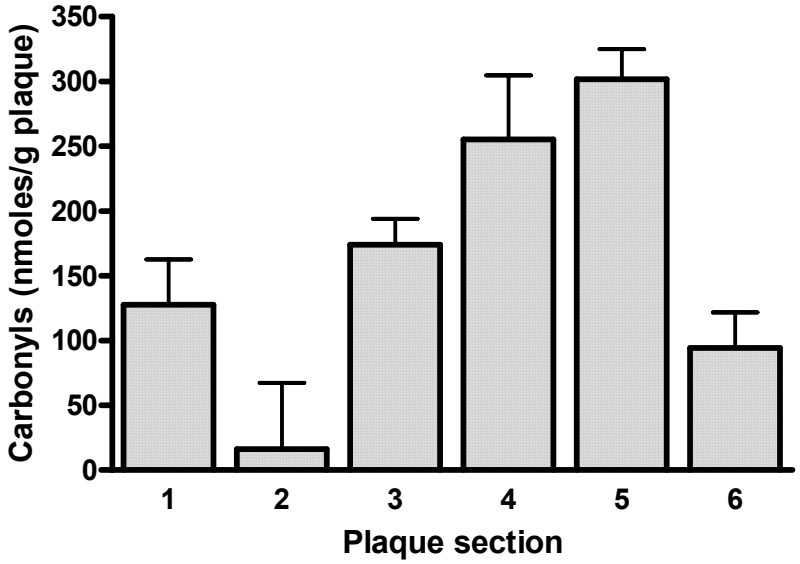


Figure 6.26 Concentration of carbonyls in six sections of plaque 1.
After dissecting the right internal carotid artery into six sections, each piece was analyzed by spectrophotometer for protein carbonyls. Results are displayed as mean ± SEM of triplicates.

Any trends observed along the atherosclerotic lesion, when measuring the protein oxidation products per gram of plaque, remained even after expressing these oxidation markers per gram of protein. By contrast, the trends for TBARS and vitamin E appeared to depend on the mode of data expression, with variation existing between per gram of plaque and per mole of cholesterol analyses. Both normalizations have therefore been used to present the TBARS and vitamin E results for plaque 1 and plaque 2.

TBARS concentrations in the six sections of plaque 1 only increased significantly ($p<0.001$) in the final two segments when expressing data per gram of plaque (Figure 6.27a). This resulted in peak TBARS concentrations of 8.77 ± 0.57 nmoles/g plaque and 8.39 ± 0.32 nmoles/g plaque for sections five and six, respectively. However, when compared on a micromole per mole of cholesterol basis, section one was associated with the highest TBARS concentration and differed significantly ($p<0.001$) from all other sections (Figure 6.27b). The subsequent decrease was only reversed to a significant extent at section five ($p<0.01$), with a further increase at section six ($p<0.001$) to a concentration that represented 77% of the TBARS content in the first segment.

When expressed per gram of plaque, the trend for vitamin E across the six sections of plaque 1 displayed similarity with the pattern of cholesterol deposition (Figure 6.23 & Figure 6.28a). A slow rise in the content of vitamin E reached significance only at section four ($p<0.001$) and was followed by a 1.9-fold increase, to 47.31 ± 0.81 nmole/g plaque, in section five ($p<0.001$). The subsequent decrease in vitamin E at section six ($p<0.001$), nevertheless, remained significantly higher than the concentrations of vitamin E in the first three sections ($p<0.001$) and even in section four ($p<0.01$).

Given the similarity between the cholesterol and vitamin E trends, this latter pattern largely disappeared when expressing vitamin E per mole of cholesterol (Figure 6.28b). Though not significant, the first three sections now showed a tendency to decrease. Furthermore, the segment containing the highest quantity of vitamin E per mole of cholesterol was identified as section four, while sections five and six were no longer significantly different from section one or each other.

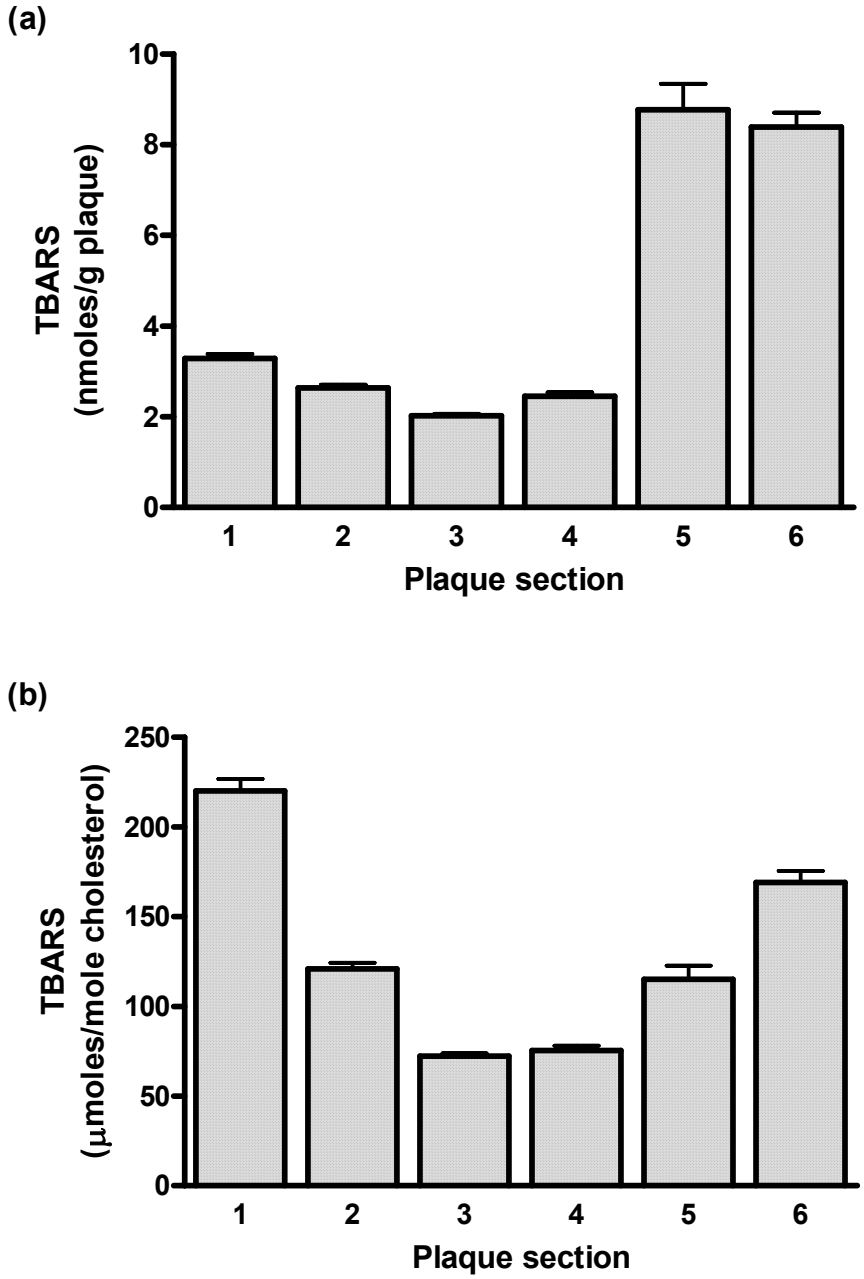


Figure 6.27 Concentration of TBARS in six sections of plaque 1.
After dissecting the right internal carotid artery into six sections, each piece was analyzed by HPLC for TBARS. Data is expressed as (a) nanomoles per gram of plaque and (b) micromoles per mole of cholesterol. Results are displayed as mean ± SEM of triplicates.

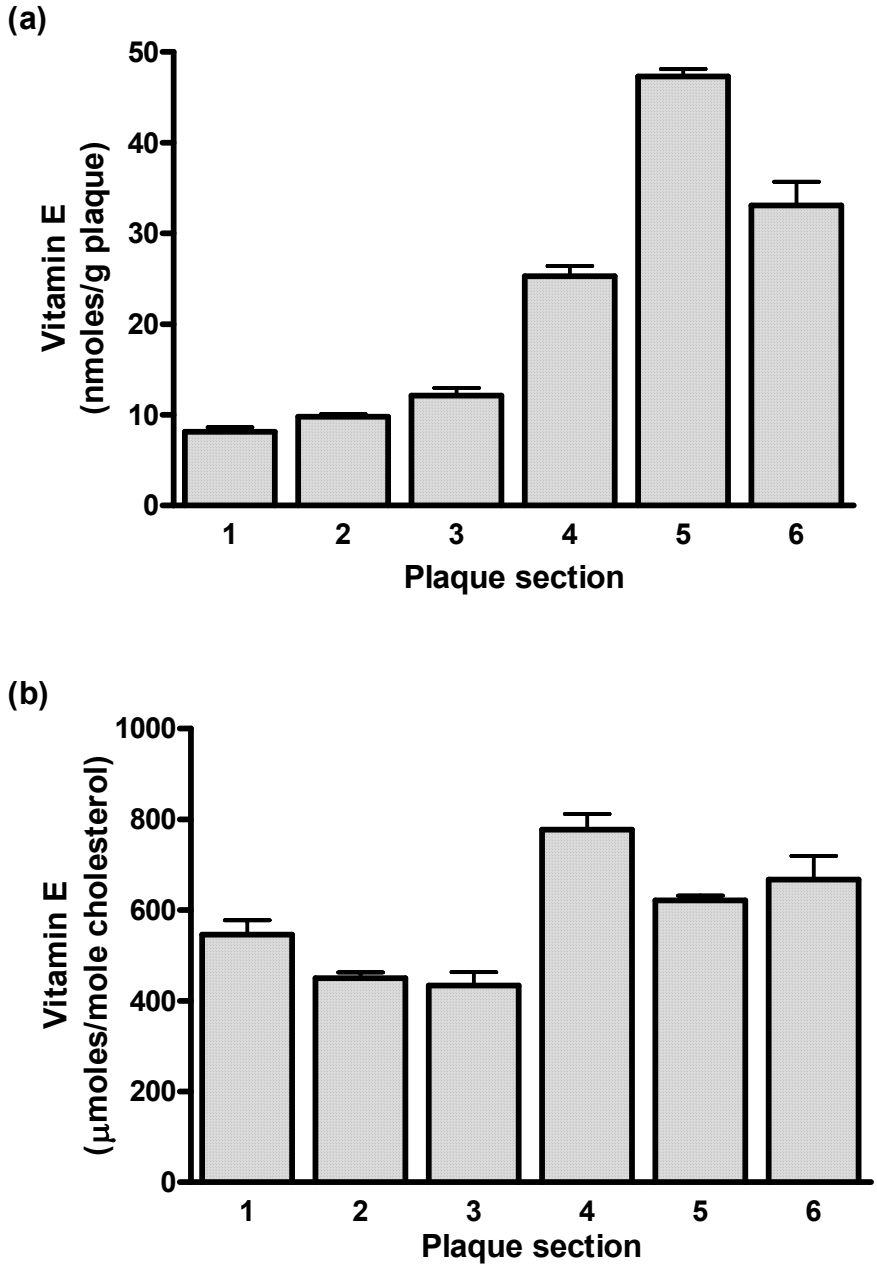


Figure 6.28 Concentration of vitamin E in six sections of plaque 1.
After dissecting the right internal carotid artery into six sections, each piece was analyzed by HPLC for vitamin E. Data is expressed as (a) nanomoles per gram of plaque and (b) micromoles per mole of cholesterol. Results are displayed as mean ± SEM of triplicates.

6.2.2.2 Trends in the Carotid Plaque from Patient 2

Approximately 39mm of plaque was removed from the right internal carotid artery of patient 2 (Figure 6.29). As this was larger than the plaque from patient 1, it was divided into seven sections, each representing 5-6mm of the plaque length and 0.268-0.654g of plaque weight.

Similar to plaque 1, parameters of oxidation and neopterin production were once again investigated.

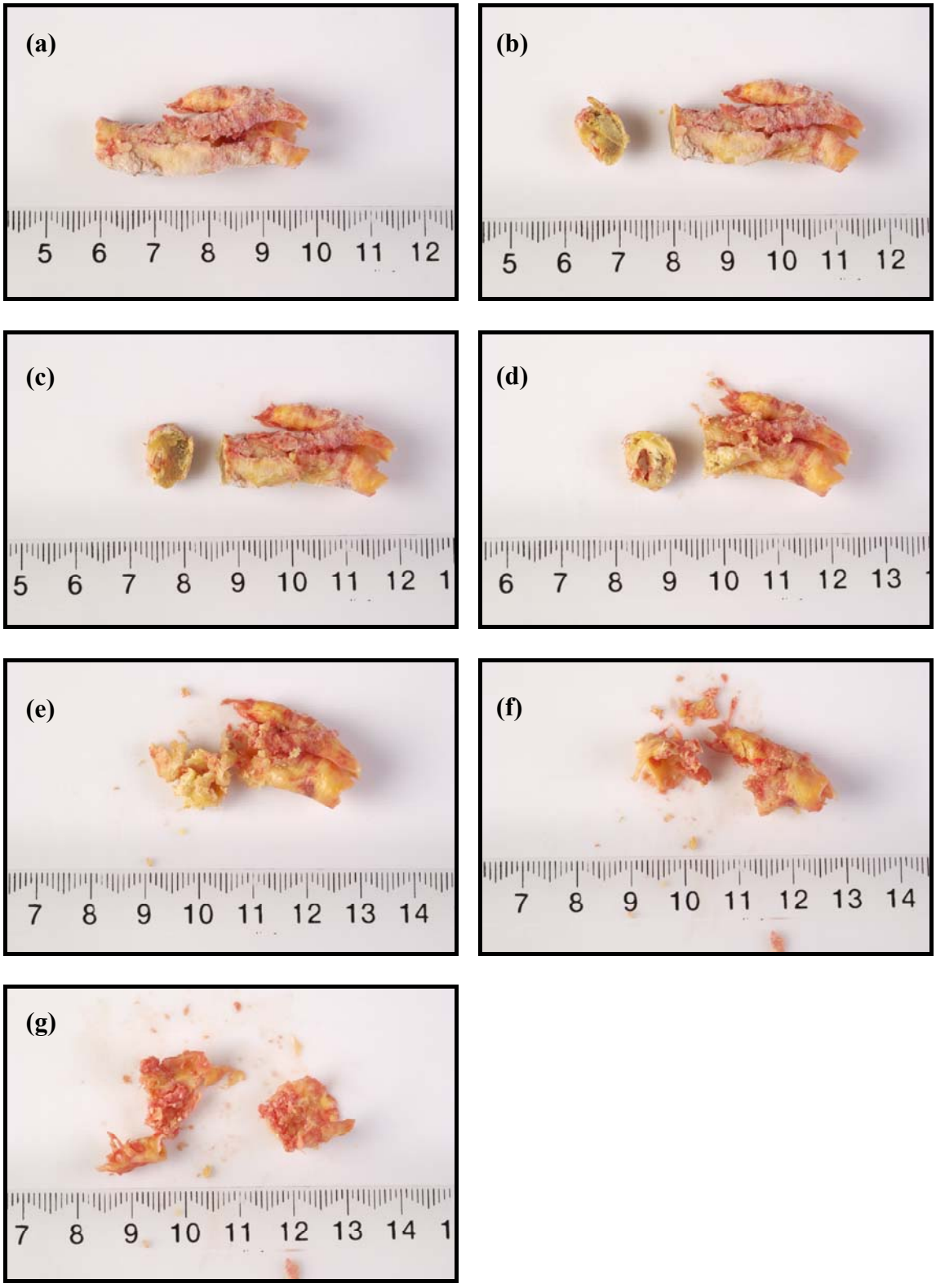


Figure 6.29 Cross-sections of the internal carotid artery from patient 2.
The (a) right internal carotid artery was removed from patient 2 and dissected into seven segments, labelled (b) section one, (c) section two, (d) section three, (e) section four, (f) section five, (g) section six (left) and section seven (right). The sixth section includes the bifurcation.

i) Neopterin

When evaluated across all sections, the neopterin (including oxidized 7,8-NP) in plaque 2 averaged 0.19 ± 0.02 nmole/g plaque (Figure 6.30). Although a similar mean was calculated for plaque 1 (Figure 6.20), the seven segments of plaque 2 were associated with a significantly smaller range of neopterin concentrations. This range varied only between 0.14-0.27 nmole/g plaque rather than 0.06-0.71 nmole/g plaque.

Neopterin peaked in section two of plaque 2 and, given a plaque density of approximately 1g/ml, the 0.27 nmole/g plaque concentration would therefore equate to a molar value of 0.27μM. The subsequent decline in neopterin levels extended to section six before rising again in section seven. However, the neopterin in this last segment still remained significantly below the peak concentration detected in section two ($p<0.001$).

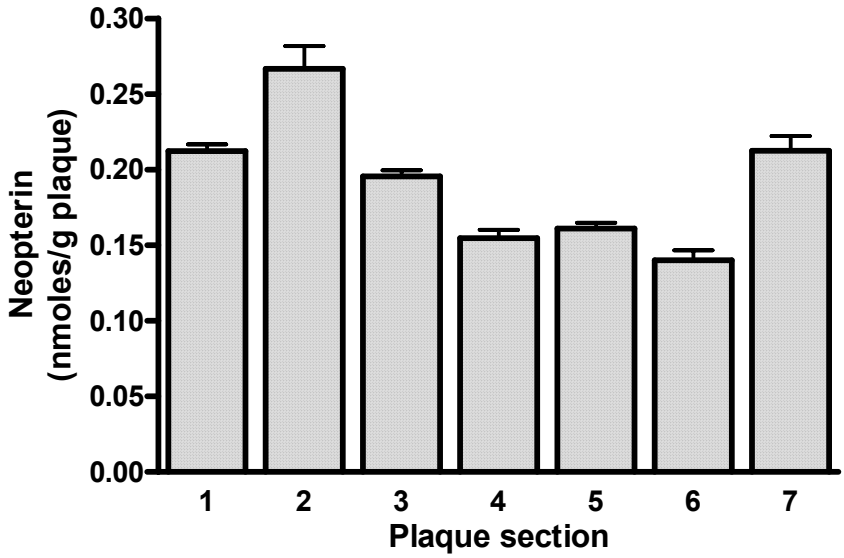


Figure 6.30 Concentration of neopterin in seven sections of plaque 2.
After dissecting the right internal carotid artery into seven sections, each section was analyzed by HPLC for neopterin (including oxidized 7,8-NP). Results are displayed as mean ± SEM of triplicates.

ii) Protein and Cholesterol

Protein appeared to be distributed along plaque 2 in a pattern similar to the one observed for neopterin (Figure 6.31). In the case of protein, however, the second rise was not as pronounced. Section seven was still significantly higher than the preceding segment ($p<0.05$) but was of a concentration more comparable to the protein content in section five rather than section one. Nevertheless, the peak protein concentration

(97.13 ± 2.24 mg/g plaque) remained associated with section two and also remained significantly elevated compared to all other regions of the plaque ($p<0.001$).

The similar distribution patterns for neopterin and protein ensured that, when expressed per gram of protein, the neopterin trend largely disappeared (data not shown). Section seven continued to be elevated ($p<0.001$) but all others, including section two, showed only minor differences in concentration from the first segment of the plaque.

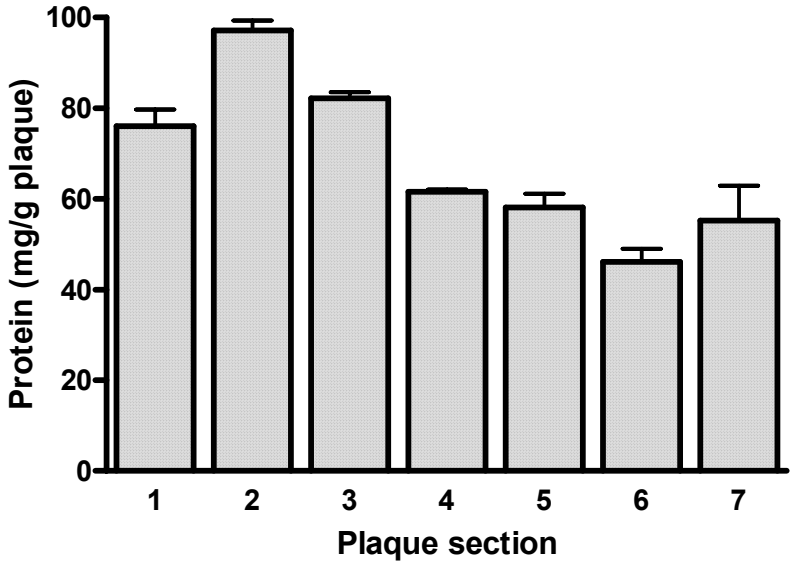


Figure 6.31 Concentration of protein in seven sections of plaque 2.
After dissecting the right internal carotid artery into seven sections, each section was analyzed by the BCA protein determination kit for protein. Results are displayed as mean ± SEM of triplicates.

Cholesterol was highest in section one ($p<0.001$) and then proceeded to progressively decline in all remaining segments (Figure 6.32). Thus, concentrations ranged from a peak of 173.9 ± 9.00 μmole/g plaque in section one to a low of 24.51 ± 1.16 μmole/g plaque by section seven. It is interesting to note that this low was, nevertheless, at a comparable level to the cholesterol concentration in four of the six sections of plaque 1 (Figure 6.23).

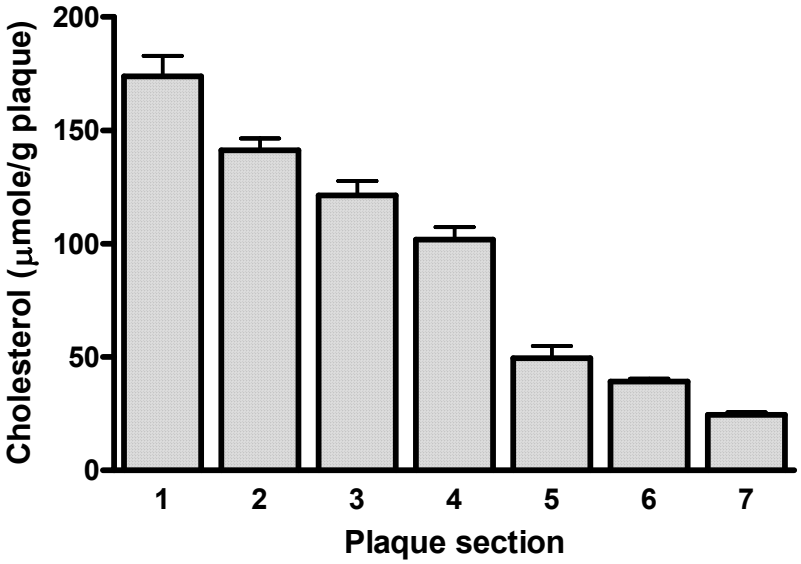


Figure 6.32 Concentration of cholesterol in seven sections of plaque 2.
After dissecting the right internal carotid artery into seven sections, each piece was analyzed by spectrophotometer for cholesterol. Results are displayed as mean ± SEM of triplicates.

iii) Markers of Oxidation

The PB-DOPA in plaque 2 displayed a tendency to decrease from the peak concentration, of 15.16 ± 0.43 nmole/g plaque, in section one (Figure 6.33). By section four, this gradual decline had reached significance ($p<0.01$), contrasting with the lack of any obvious PB-DOPA trend for plaque 1 (Figure 6.24). One exception to this general pattern of reduction was noted in the penultimate section of plaque 2, with a brief increase in PB-DOPA that was lower than, though not significantly different from, the peak concentration in section one.

While PB-DOPA levels were dropping, dityrosine increased significantly ($p<0.001$) in the first three segments of plaque 2 (Figure 6.34). The peak concentration of 14.64 ± 0.53 nmole/g plaque in section three then dropped dramatically ($p<0.001$) and this decline continued, ultimately resulting in a concentration of 0.49 ± 0.09 nmole/g plaque in section seven. Interestingly, four of the six sections of plaque 1 actually possessed a smaller amount of dityrosine than the minimum quantity detected in plaque 2 (Figure 6.25).

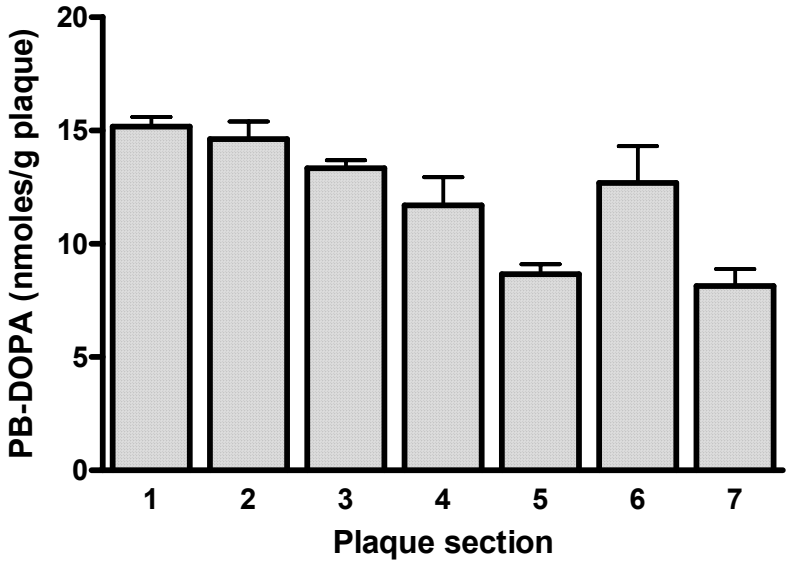


Figure 6.33 Concentration of PB-DOPA in seven sections of plaque 2.
After dissecting the right internal carotid artery into seven sections, each piece was analyzed by HPLC for PB-DOPA. Results are displayed as mean ± SEM of triplicates.

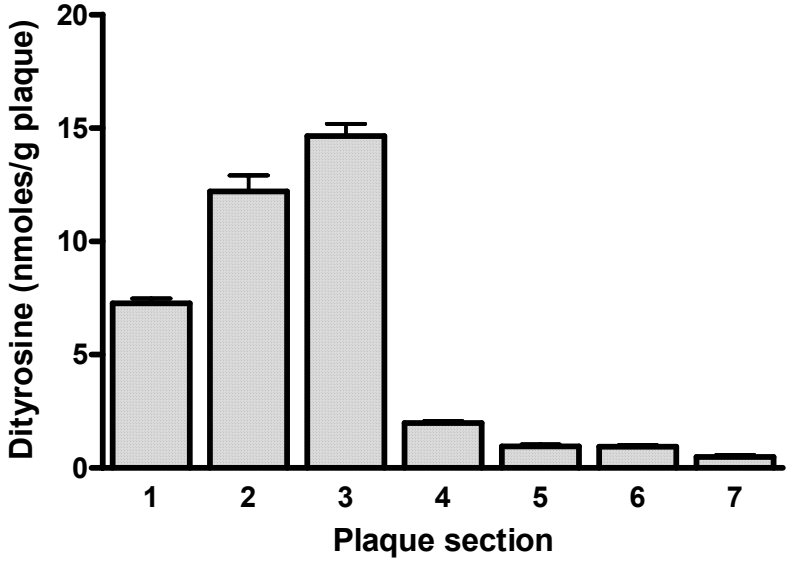


Figure 6.34 Concentration of dityrosine in seven sections of plaque 2.
After dissecting the right internal carotid artery into seven sections, each piece was analyzed by HPLC for dityrosine. Results are displayed as mean ± SEM of triplicates.

Carbonyls were a more abundant protein oxidation product in each section of plaque 2 when compared to PB-DOPA and dityrosine (Figure 6.33 to Figure 6.35). The carbonyls of plaque 2 were also generally more concentrated than the carbonyls of plaque 1 (Figure 6.26 & Figure 6.35). Four of the six sections from the first plaque

were found to contain less than the minimum plaque 2 carbonyl concentration of 217.1 ± 21.70 nmole/g plaque.

Although the carbonyl concentration increased and decreased a couple times along the length of plaque 2, no strong trend was apparent (Figure 6.35). Indeed, the final three sections possessed levels of carbonyls that were not significantly altered from the first segment. Even expressing carbonyls in micromoles per gram of protein failed to introduce any clearly identifiable pattern of distribution (data not shown).

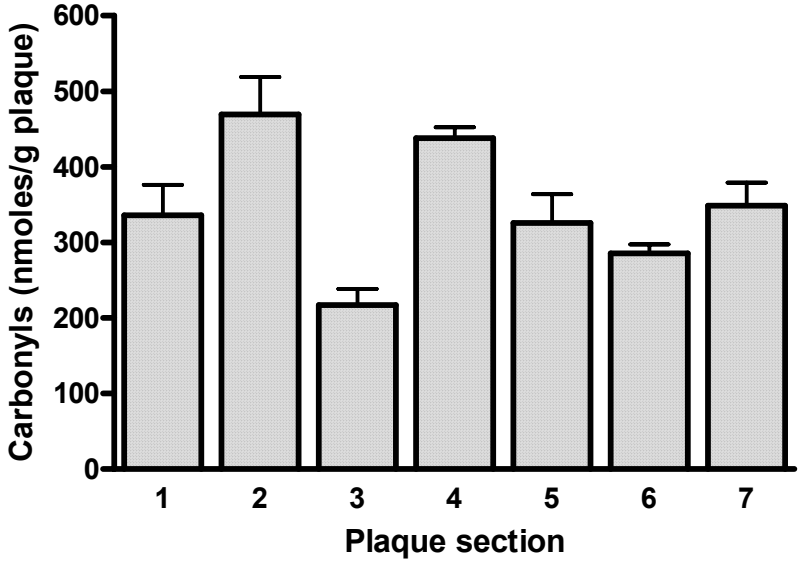


Figure 6.35 Concentration of carbonyls in seven sections of plaque 2.
After dissecting the right internal carotid artery into seven sections, each piece was analyzed by spectrophotometer for protein carbonyls. Results are displayed as mean ± SEM of triplicates.

TBARS were found to be most concentrated in the first two sections of plaque 2 when expressing the data per gram of plaque (Figure 6.36a). The subsequent decline from this peak of 6-7 nmole/g plaque extended to section six before rising slightly again in section seven. Although this final increase was significant compared to the preceding section ($p<0.001$), it nevertheless produced a concentration that represented only 42% of the peak TBARS level. Such a pattern contrasts with plaque 1, which contained the highest quantity of TBARS in its final two sections (Figure 6.27a). This trend for plaque 2 also contrasts with the results obtained after normalizing all TBARS data against cholesterol (Figure 6.36b). In this case, levels remained low throughout the first six sections but increased by 2.7-fold in section seven ($p<0.001$).

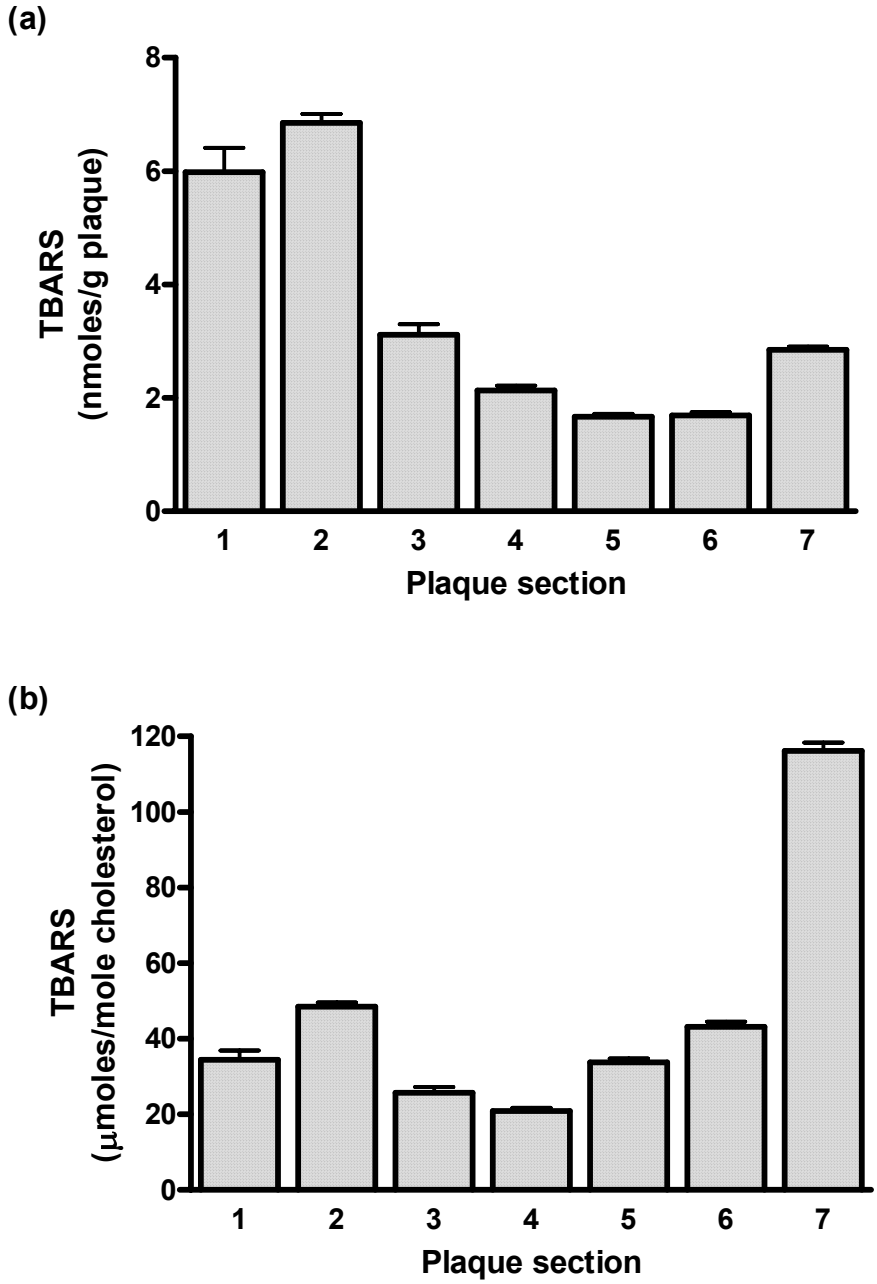


Figure 6.36 Concentration of TBARS in seven sections of plaque 2.
After dissecting the right internal carotid artery into seven sections, each piece was analyzed by HPLC for TBARS. Data is expressed as (a) nanomoles per gram of plaque and (b) micromoles per mole of cholesterol. Results are displayed as mean ± SEM of triplicates.

While plaque 1 was associated with no obvious pattern of vitamin E distribution after normalization against cholesterol (Figure 6.28b), this lack of trend was only noted in plaque 2 when expressing the antioxidant in nanomoles per gram of plaque (Figure 6.37a). Although the final segment was lower than sections two and four ($p<0.001$), it was not significantly different from the remaining four sections. A trend did appear,

however, when expressing the vitamin E of plaque 2 in micromoles per mole of cholesterol (Figure 6.37b). This data analysis revealed a general tendency for the vitamin E to increase along the length of the plaque and is similar to the distribution of vitamin E in plaque 1 when measured on a per gram of plaque, but not on a per mole of cholesterol, basis (Figure 6.28).

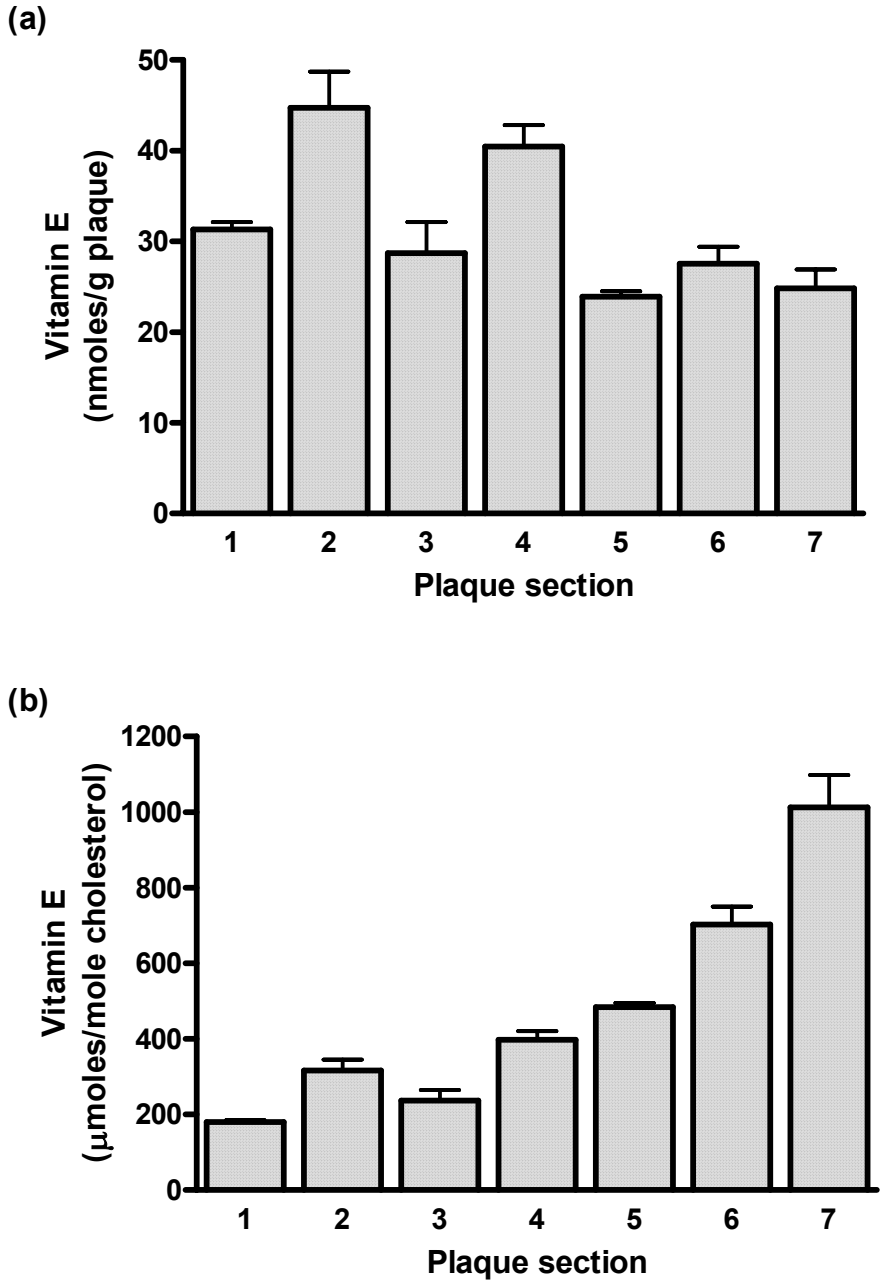


Figure 6.37 Concentration of vitamin E in seven sections of plaque 2.
After dissecting the right internal carotid artery into seven sections, each piece was analyzed by HPLC for vitamin E. Data is expressed as (a) nanomoles per gram of plaque and (b) micromoles per mole of cholesterol. Results are displayed as mean ± SEM of triplicates.

6.2.2.3 Preliminary Correlations Between Markers of Oxidation and Inflammation Along the Length of the Carotid Plaque

This investigation ultimately aims to compare and identify patterns along the length of the plaques and also between the various oxidation parameters. Although the current pilot study is too small to permit such trends and correlations to be identified conclusively, a few initial associations have been highlighted in Table 6.4. It should be noted, however, that these correlations appeared to differ between the two patients and to differ, yet again, once the data from both patients was combined. Only the dityrosine/protein and cholesterol/TBARS relationships were common to the two plaques but even these disappeared during an analysis of the pooled plaque data.

The correlations in Table 6.4 were identified using data expressed per gram of plaque. When analyzed on a per protein or per cholesterol basis, many of the Table 6.4 associations were lost and new ones appeared (data not shown). However, given the small sample size in this pilot study, these preliminary correlations will not be further discussed.

Table 6.4 Correlations between markers of oxidation and inflammation along the length of plaque.

Plaque samples from patient 1 and patient 2 were cut into six and seven sections, respectively. Each piece was then analyzed for the content of neopterin, protein, cholesterol, PB-DOPA, dityrosine, carbonyls, TBARS and vitamin E. Relationships between these parameters were subsequently investigated for each individual patient and for the two patients combined, with the data expressed as per gram of plaque. All the listed associations are positive and significant, though the level of significance may vary and is indicated by * for $p<0.05$; ** for $p<0.01$; or *** for $p<0.001$.

Correlation	p value	r^2 (Pearson)
Patient 1		
Dityrosine vs. Protein	0.019 (*)	0.782
Dityrosine vs TBARS	0.023 (*)	0.762
Cholesterol vs. TBARS	0.025 (*)	0.752
Cholesterol vs Vitamin E	0.001 (**)	0.946
TBARS vs. Vitamin E	0.029 (*)	0.737
Patient 2		
Neopterin vs. Protein	0.025 (*)	0.668
Neopterin vs. TBARS	0.008 (**)	0.784
Carbonyl vs. Vitamin E	0.041 (*)	0.601
PB-DOPA vs Cholesterol	0.016 (*)	0.718
Dityrosine vs. Protein	0.005 (**)	0.816
Protein vs. Cholesterol	0.028 (*)	0.655
Protein vs. TBARS	0.020 (*)	0.696
Cholesterol vs. TBARS	0.036 (*)	0.619
Combined Patient Data		
Carbonyl vs. Cholesterol	0.042 (*)	0.325
Carbonyl vs. Vitamin E	0.008 (**)	0.489
Dityrosine vs. Cholesterol	0.001 (**)	0.627
Protein vs. TBARS	0.003 (**)	0.569
Cholesterol vs. Vitamin E	0.018 (*)	0.415
TBARS vs. Vitamin E	0.027 (*)	0.372

6.3 Discussion

6.3.1 Neopterin in Pus

The pus removed from sites of inflammation predominantly constitutes cellular debris, leukocytes and plasma infiltrate (Dox *et al.*, 1993). As such, this inflammatory material is expected to contain significantly higher concentrations of pterin than serum or urine. Healthy people are generally associated with levels of serum neopterin below 10nM (Tatzber *et al.*, 1991) while people suffering from infections or various inflammatory diseases may contain slightly, though significantly, elevated serum neopterin concentrations. In some cases this elevation can be quite pronounced, with quantities reaching 0.1μM neopterin in the serum of one or two HIV (Fuchs *et al.*, 1994) and atherosclerosis (Tatzber *et al.*, 1991) patients. By contrast, the neopterin in pus averaged a concentration of 0.51 ± 0.07μM (Table 6.2), thereby confirming that levels are generally higher at the site of inflammation than in serum. Furthermore, the peak concentration in this inflammatory material, of 1.28μM neopterin, falls within a range comparable to the concentrations of 7,8-NP required to delay copper- and THP-1 monocyte-mediated LDL oxidation (Gieseg *et al.*, 1995; Gieseg & Cato, 2003). It remains possible that microenvironments may exist in the inflammatory material and that these may be associated with even higher levels of neopterin.

While one study detected no significant gender difference in serum neopterin (Maloney *et al.*, 1997), several others have noted an increased concentration in females compared to males (Weiss *et al.*, 1994; Garcia-Moll *et al.*, 2000a). This gender distinction remained whether investigating neopterin in healthy subjects or CAD patients. The apparent discrepancy with the first study may simply be due to differences in sample size. Maloney *et al.* (1997) analyzed 135 healthy subjects but the equivalent subset in the Weiss *et al.* (1994) study contained 240 people. Sample size may also account for the fact that, although a trend towards higher neopterin concentrations in female pus was identified, it failed to reach significance (Figure 6.1).

Like gender, age has also been suggested to affect the quantity of neopterin in serum. Indeed, the positive correlation identified between these two parameters was noted in sample groups ranging from insulin-resistant subjects and CAD patients to healthy individuals (Ledochowski *et al.*, 1999; Schennach *et al.*, 2002; Tanaka *et al.*,

2004; Avanzas *et al.*, 2005; Murr *et al.*, 2005). By contrast, one study of 135 healthy people (Maloney *et al.*, 1997) and another using 37 haemodialysis patients (Erten *et al.*, 2005), failed to detect any association between serum neopterin and age. Likewise, neopterin did not appear to significantly increase with age in the 19 pus samples analyzed (Figure 6.2). While these apparent discrepancies may be caused by limitations in the sample size, the age range of the subjects should also be taken into account. It has been suggested that serum neopterin may not increase much between the ages of 18 and 75, but it is significantly lower in this age group than in people aged over 75 years (Hamerlinck, 1999). Given that only two of the pus samples were removed from patients older than 75, such differences would not have been adequately highlighted in the current study. A further point of consideration regards the theory that neopterin levels become elevated as people age due to the development of underlying, and unidentified, inflammatory disease processes (Ledochowski *et al.*, 1999; Murr *et al.*, 2005). As neopterin in the blood is derived from multiple sources, this elevation would ultimately be detectable in the serum. By contrast, the pus neopterin measured in the current study is produced at a local site of inflammation. It is therefore not likely to be affected by the more distant inflammatory events associated with aging.

Neopterin is often considered to be a marker of immune activation, raising the possibility of a correlation between this pteridine and either macrophages or white cells in the bloodstream. Such associations were not significant in the current study (Figure 6.3). Nevertheless, a positive trend between neopterin in the pus and white cells in the blood was noted, and appeared to be approaching significance ($p=0.096$). Given a sample size larger than 19, a significant correlation may therefore have been detectable. Others have tried to identify similar correlations when monitoring serum neopterin. One study, involving 50 patients with unstable angina, failed to detect any relationship between these parameters (Garcia-Moll *et al.*, 2000a). Meanwhile, another group investigated individuals with truncal obesity and did observe a positive correlation between serum neopterin and leukocyte levels in the blood (Bozdemir *et al.*, 2006).

Numerous studies have investigated the existence of additional associations with neopterin. For instance, body mass index (Ledochowski *et al.*, 1999; Bozdemir *et al.*, 2006), immune activation and metabolism (Erren *et al.*, 1999) and the severity/extent of various diseases or infections (Hamerlinck, 1999; Hoffmann *et al.*, 2003) have all been monitored against serum neopterin. However, none have attempted to identify the relationship between markers of oxidation and neopterin at the site of inflammation.

An attempt to remedy this lack of knowledge by analyzing pus revealed that neopterin did not exhibit any significant association with either protein content (Figure 6.7) or products of protein oxidation (Figure 6.4 to Figure 6.6). Likewise, no correlation was detectable between neopterin and a lipid oxidation product like TBARS (Figure 6.8). These findings do not provide support for pterins serving as antioxidants at sites of acute inflammation. The absence of such correlations could certainly be explained by the relationships never existing. Alternatively, the correlations may have been present during early stages of inflammation but been subsequently overwhelmed during the advanced stages when the pus was finally removed.

In contrast to the lack of correlation between neopterin and markers of lipid and protein oxidation, an association was able to be identified with vitamin E (Figure 6.9). However, even this was only significant when both parameters were expressed relative to protein rather than on a molar basis. The reason for this positive association with a well-known antioxidant is not known, but it is tempting to speculate that both may be fulfilling a similar function *in vivo*. Nevertheless, it should be noted that the average neopterin concentration in the pus of 0.51 ± 0.07μM is significantly lower than the average vitamin E concentration of 23.07 ± 3.04μM (Table 6.2). Both compounds are suggested to scavenge peroxyl radicals at a comparable rate (Oettl *et al.*, 1997). Neopterin would therefore need to act at a different site, or be significantly elevated in a microenvironment, if it were to play any significant antioxidant role compared to vitamin E in the inflammatory surroundings of pus. Neither possibility was able to be investigated in the pus due to the method of isolation and subsequent homogenization of this inflammatory material.

6.3.2 Comparisons Between Markers of Oxidation in Pus

PB-DOPA, dityrosine, protein carbonyls, TBARS and vitamin E were all shown to be components of pus. Potential relationships between these different oxidation parameters could therefore be investigated. An increase in protein and lipid oxidation products was associated with increased pus protein content (Figure 6.10 to Figure 6.12). Thus, as expected, the concentration of inflammatory material strongly influences the extent of oxidation detected in the pus.

A positive relationship between PB-DOPA and dityrosine arises as a consequence of their common source of origin (Figure 6.13). Both are products of tyrosine oxidation,

with PB-DOPA generated via hydroxylation of a single tyrosine residue and dityrosine formed by the cross-linkage of two tyrosyl radicals (Davies *et al.*, 1999). Compared to PB-DOPA, the production of dityrosine is therefore more dependent on the rate of radical attack. It also requires two tyrosine residues to reside in close proximity. These limitations explain why PB-DOPA predominates over dityrosine in pus (Table 6.2) and in other inflammatory environments, including atherosclerotic plaque (section 6.3.4; Fu *et al.*, 1998; Woods *et al.*, 2003).

PB-DOPA is clearly a more common product of oxidation than dityrosine but is, conversely, significantly less abundant than protein carbonyls. Carbonyls in the pus were, on average, 10-20 times more concentrated than PB-DOPA (Table 6.2). Unlike the two markers of tyrosine oxidation, carbonyls are often present as native components of protein, and their formation under oxidative conditions is not restricted to a single amino acid (Dalle-Donne *et al.*, 2003). Lipid- and sugar-derived aldehydes/ketones are also suggested to contribute to the total concentration of protein-associated carbonyls by binding to this protein substrate in secondary reactions (Burcham & Kuhan, 1996; Davies *et al.*, 1999).

PB-DOPA and dityrosine not only correlated with each other in the inflammatory environment of the pus but also both shared a relationship with TBARS (Figure 6.14 & Figure 6.15). Thus, the increase in oxidative damage to protein appears to be paralleled by an increase in lipid peroxidation. It is important to note, however, that these relationships are strongly dependent on the protein content of the pus. When PB-DOPA, dityrosine and TBARS were expressed relative to protein, all correlations disappeared (data not shown). These correlations also lost significance if the outlier attributed to patient 2 was removed from analysis. While this does raise concerns regarding the strength of the above associations, these findings can still be considered to provide a useful starting point for further studies.

In direct contrast to the lack of a relationship between the putative pterin antioxidant and oxidized protein or lipid, vitamin E was confirmed to negatively correlate with both PB-DOPA and TBARS (Figure 6.16 & Figure 6.17). Although vitamin E is a lipid soluble antioxidant, and might therefore be expected to inhibit the formation of TBARS more effectively than PB-DOPA, these two associations were actually of a similar strength ($p<0.05$, $r^2=0.212$). Consequently, the potential exists for lipid-derived radicals to participate in the generation of PB-DOPA and for the vitamin E to scavenge these

radicals and, thus, prevent TBARS as well as PB-DOPA from being produced. A similar theory of PB-DOPA formation was proposed to explain the close correlation observed between this tyrosine oxidation product and the generation of conjugated dienes during AAPH-mediated LDL oxidation (Gieseg *et al.*, 2003). Further support for this theory cannot be gained from the current study. As discussed above, a positive association between TBARS and PB-DOPA, that was independent of the protein content in pus, could not be confirmed.

Although vitamin E was negatively associated with the concentration of TBARS and PB-DOPA, this was not the case for the other oxidation markers analyzed in pus. The lack of a correlation between vitamin E and dityrosine may simply be due to this oxidation product being generated independently of the lipid-derived radicals that the antioxidant scavenges. Furthermore, dityrosine was generally present only at low levels in the pus (interquartile range of 0.09-0.57μM), and this would have severely limited any opportunity to observe such a correlation during the analysis of only 19 subjects.

The relationship between vitamin E and protein carbonyls was neither neutral nor negative but, unexpectedly, positive (Figure 6.18). It has been suggested that, in the presence of a sufficiently low radical flux, vitamin E can actually exhibit pro-oxidant activities (Bowry *et al.*, 1992; Neuzil *et al.*, 1997). Although this would certainly explain the positive association between vitamin E and carbonyls, the negative correlations with TBARS and PB-DOPA suggest that a pro-oxidant function for vitamin E is not a general feature of the current study. Carbonyls are not simply markers of oxidation but also native components of protein. However, the correlation between carbonyls and vitamin E could not be accounted for by an increase in the protein content of pus. The relationship remained both positive and significant even when carbonyls and/or vitamin E were expressed per gram of protein. Carbonyls have been described as reasonably stable markers of oxidation (Dalle-Donne *et al.*, 2003) but may still, ultimately, undergo further reactions (Davies *et al.*, 1999). Aldehydes may oxidize to carboxylic acids while both aldehydes and ketones can react with amines in Schiff base reactions, followed by irreversible rearrangements to Amadori products. Perhaps these reactions are less favourable in environments associated with higher concentrations of antioxidant, thereby resulting in an apparently higher carbonyl concentration and a positive association with vitamin E.

6.3.3 Neopterin in Plaque

The atherosclerotic plaque is a site of chronic inflammation, representing a likely source of macrophages that are both activated and capable of synthesizing pterin. As a consequence, neopterin is expected to be more concentrated in plaque than in serum. While numerous studies have analyzed neopterin in the serum, and identified a positive relationship with atherosclerosis (Tatzber *et al.*, 1991; Weiss *et al.*, 1994; Gurfinkel *et al.*, 1999; Erten *et al.*, 2005), none attempted to study levels within the atherosclerotic plaque. The current pilot study has therefore attempted to resolve this gap in knowledge, using carotid plaque removed from two donors. By dividing the plaques into sections, it has also been possible to determine whether levels vary or remain relatively constant throughout the plaque.

Neopterin was, indeed, detected in all 13 sections of plaque analyzed, resulting in an average concentration of 0.19 nmole/g plaque for each patient (Figure 6.20 & Figure 6.30). Further analysis of each section from patient 1 revealed relatively low (0.06-0.12 nmole/g plaque), but consistent, quantities of neopterin in all but one segment. The exception, section four, contained a peak concentration of 0.71 nmole/g plaque. This is approximately equivalent to 0.71μM neopterin and, although smaller than the maximum pus neopterin level of 1.28μM, it still approaches a concentration in the micromolar range. Just as critically, these findings confirm that the quantity of neopterin is not necessarily homogeneous along the length of the plaque. This makes sense considering that macrophages are also known to be distributed unevenly throughout the plaque (Bjorkerud & Bjorkerud, 1996; Scott, 2004). These findings therefore also suggest that microenvironments, containing even higher quantities of neopterin, may exist within certain regions of the plaque.

In contrast to patient 1, the seven sections of plaque from patient 2 were associated with a much smaller range of neopterin concentrations (0.14-0.27 nmole/g plaque). Thus, although neopterin peaked at a significantly elevated level in section two compared to the remaining segments, this increase was not as dramatic as that observed in plaque 1. A sample size of two is, admittedly, insufficient for making conclusive claims regarding neopterin's deposition along the length of the inflammatory site. Nevertheless, this pilot study does indicate that the pattern of neopterin localization in plaque is not necessarily comparable between patients.

While the patients shared similar demographic data, one major difference was the medication being prescribed prior to hospital admission for the carotid endarterectomy.

This is of relevance because aspirin (Schroecksnadel *et al.*, 2005) and Atorvastatin (Neurauter *et al.*, 2003) have been reported to inhibit neopterin formation in IFN-γ-simulated macrophages. A clinical study has also shown serum neopterin levels to transiently decrease in 10 subjects taking Fluvastatin (van Haelst *et al.*, 2001), while a couple of larger studies observed that statins in general were associated with lower serum neopterin levels than non-medicated controls (Walter *et al.*, 2003; Avanzas *et al.*, 2005). Both patients were prescribed aspirin but only patient 2 was taking a statin, Atorvastatin (Table 6.3). It is therefore possible that the absence of a large neopterin spike in this second patient is due to the anti-inflammatory, neopterin-lowering effects of the statin. Larger samples are clearly needed to more adequately investigate this theory.

The small sample size also prevents neopterin levels in the plaque and pus from being conclusively compared. Having acknowledged this, it is interesting to note that the average neopterin concentration in the two atherosclerotic plaques is approximately 40% of the average detected in pus. Such observations hint at a potential difference between the chronic inflammatory environment of the plaque and the acute inflammation in the pus. At the late stage of atherosclerosis, when the carotid plaques were surgically removed, many of the macrophages may have been so lipid-loaded and close to death that pterin synthesis was no longer a significant aspect of macrophage metabolism. Furthermore, neopterin production is inhibited in IFN-γ-activated macrophages exposed to iron (Weiss *et al.*, 1992) and the inflammatory mediator histamine (Gruber *et al.*, 2000). Both have been suggested to be present in plaque, particularly at the advanced stages of the disease (Smith *et al.*, 1992; Lamb *et al.*, 1995; Swain & Gutteridge, 1995; Sasaguri & Tanimoto, 2004; Stadler *et al.*, 2004).

In addition to monitoring neopterin's trend along the length of the plaque, it will also ultimately be possible to investigate the existence of correlations between this pterin and various markers of oxidation. Identifying these associations was of limited success in the current study, due both to the small number of patients sampled and the small number of sections obtained for each plaque. Plaque 1 yielded no significant relationships while the neopterin in plaque 2 correlated positively with protein and TBARS concentrations when expressed per gram of plaque (Table 6.4). Confirming or denying the general nature of such correlations will require a sample size much greater than the two patients analyzed in the pilot study.

6.3.4 Markers of Oxidation in Plaque

The plaque was homogenized using a procedure previously described by Fu *et al.* (1998). This group confirmed that amino acids were not oxidized as a consequence of the homogenization protocol. Thus, oxidation is not expected to be an artefact of homogenization in the current study either.

As discussed above, the pattern of neopterin localization along the length of the plaque was not comparable between the two patients investigated in the current study (section 6.3.3). It soon became apparent that this was also the case for each marker of oxidation. However, whether such observations are a general feature of atherosclerotic plaques will only be known once a larger number of samples have been analyzed.

The small sample size has, likewise, prevented correlations between the various markers of oxidation in plaque from being reliably identified. Those that appeared in the plaque of patient 1 were generally not present in the plaque from patient 2 (Table 6.4). This lack of similarity was observed whether measuring PB-DOPA, dityrosine, carbonyls and vitamin E relative to plaque weight, protein weight or mole of cholesterol.

Protein was at a similar level in both patients, with an average concentration across the sections of 77.62 ± 8.05 mg/g plaque and 68.06 ± 6.72 mg/g plaque for patient 1 and 2, respectively (Figure 6.22 & Figure 6.31). This is comparable to the 55.1 mg/g plaque determined for protein in an earlier study (Suarna *et al.*, 1995). Despite similar averages, however, it should be noted that the protein trend along the plaque was slightly different for each patient.

Trends were also dissimilar for cholesterol concentration, with a tendency to increase towards the bifurcation in plaque 1 but to decrease in the same direction of plaque 2 (Figure 6.23 & Figure 6.32). Suarna *et al.* (1995) did not investigate the cholesterol content along the length of the plaque but did determine that, on average, free cholesterol was the major lipid component associated with the atherosclerotic material of eleven carotid endarterectomy patients. These same subjects had free cholesterol concentrations ranging between 0.12 and 1.27mmole/g protein. Although the cholesterol assay used in the current study measures both free and esterified forms, the average quantity of cholesterol across all sections of plaque 1 and 2 fell within this range. Considering that free cholesterol is the predominant lipid component in plaque, this finding is not unexpected and it reduces the concern that any products of lipid oxidation were significantly interfering in the cholesterol assay. With a mean

cholesterol concentration of 0.47 ± 0.11 mmole/g protein (37.15 ± 9.15 µmole/g plaque) and 1.29 ± 0.23 mmole/g protein (93.05 ± 21.39 µmole/g plaque) for plaque 1 and 2, respectively, it is also apparent that the latter plaque contains an average of 2.5-fold more cholesterol than the former. This agrees with the generally more gruel-filled appearance noted for the sections of plaque removed from patient 2 compared to patient 1 (Figure 6.19 & Figure 6.29).

Products of protein oxidation have been previously detected by other groups in homogenized atherosclerotic plaque (Leeuwenburgh *et al.*, 1997b; Fu *et al.*, 1998; Upston *et al.*, 2002; Woods *et al.*, 2003). The current study extended these findings by demonstrating the existence of PB-DOPA and carbonyls in every section of plaque analyzed (Figure 6.24; Figure 6.26; Figure 6.33; Figure 6.35). Even dityrosine was present in all but one segment (Figure 6.25 & Figure 6.34).

Both pus (section 6.2.1.2) and atherosclerotic plaque (Fu *et al.*, 1998; Woods *et al.*, 2003) were found to contain PB-DOPA at significantly higher concentrations than dityrosine. A similar pattern also tended to be observed in each of the 13 sections of plaque analyzed in this pilot study. PB-DOPA remained higher than dityrosine in all samples, only losing significance in the second and third sections of plaque 2 due to a dramatic elevation in the content of dityrosine. In further agreement with the pus data, carbonyls were significantly higher than PB-DOPA and dityrosine in each of the seven segments of plaque 2 and all but one segment of plaque 1. Reasons for this trend (carbonyls > PB-DOPA > dityrosine) were discussed in section 6.2.1.2, with respect to pus, and are likely to be just as applicable for plaque.

While investigating the presence of various oxidation markers in the atherosclerotic plaque, Wood *et al.* (2003) estimated that 83% of PB-DOPA and 96% of dityrosine were present in the extracellular matrix component (ECM) of the plaque. It seems reasonable to expect that PB-DOPA, dityrosine, and even protein carbonyls, may be similarly located in the ECM of each section of plaque analyzed in the current study.

As stated previously, the trends between the two plaques are not consistent. Nevertheless, of the three protein oxidation markers monitored, the dityrosine for both patients appeared to display the most pronounced relative change in concentration. This general observation shares some similarity with a study by Upston *et al.* (2002). They noted that most tyrosine oxidation products did not vary significantly as the lesion severity increased. Dityrosine proved to be the exception and was found to be

significantly elevated at advanced stages of atherosclerosis. The current study only analyzed advanced lesions, but it could be postulated that regions along the plaque may provide a broad analogy to lesions of differing severity.

Oxidized lipids are one of the most commonly studied components of an atherosclerotic plaque. Despite such extensive investigations, attempts to identify a relationship between lipid oxidation and the extent of atherosclerosis have yielded mixed results. One study of 30 carotid plaques found a correlation between plaque instability and hydroxyeicosatetraenoic acid (Mallat *et al.*, 1999) but a second study, analyzing 47 carotid plaques, was unable to confirm this association (Waddington *et al.*, 2003). Another group measured TBARS, rather than hydroxyeicosatetraenoic acid, in the carotid plaque of 41 endarterectomy patients. They noted a positive association between this lipid oxidation product and the likelihood of plaque instability (Nishi *et al.*, 2002). Likewise, a positive correlation was shown to exist between the quantity of cholesteryl ester hydro(pero)xides in lesions and the severity of coronary atherosclerosis (Upston *et al.*, 2002).

Relationships have even been identified between atherosclerosis and plasma-associated lipid hydroperoxides (Kovacs *et al.*, 1997) or TBARS (Bonithon-Kopp *et al.*, 1997; Salonen *et al.*, 1997). In the latter case, however, Bonithon-Kopp *et al.* (1997) observed a correlation between plasma TBARS and the extent of carotid atherosclerosis only in men. By contrast, Salonen *et al.* (1997) showed a strong association with the progression of carotid atherosclerosis by measuring TBARS associated with plasma LDL only.

Given the small sample size in the current study, it is premature to conclusively identify correlations between the concentration of TBARS and atherosclerosis. Nevertheless, TBARS was certainly detectable in every segment of plaque analyzed (Figure 6.27 & Figure 6.36). Combined with the findings from other groups, this suggests that a more complete investigation of correlations between lipid oxidation and atherosclerosis may be possible by expanding the pilot study. The concentration of TBARS along the length of the plaques could then be compared against various other markers of oxidation.

Vitamin E was confirmed to co-exist with lipid oxidation products in each of the 13 sections of carotid plaque analyzed (Figure 6.28 & Figure 6.37). This agrees with

previous plaque studies (Suarna *et al.*, 1995; Niu *et al.*, 1999; Upston *et al.*, 2002), and indicates that atherosclerotic lesions are not necessarily antioxidant deficient environments. Whether the presence of this antioxidant is a response to oxidative stress is currently unclear. It also remains possible that significant amounts of the oxidative damage may have occurred in regions lacking suitable quantities of antioxidants. Such areas would include microenvironments or sections associated with an early loss of antioxidant, followed by a subsequent re-introduction, only after the oxidative damage has occurred (Niu *et al.*, 1999). Alternatively, the plaque may represent a site of very low radical flux, thereby causing the vitamin E to exert a pro-oxidant activity (Bowry *et al.*, 1992; Neuzil *et al.*, 1997). Sampling from the plaque at a single time point makes it difficult to distinguish between such possibilities. Nevertheless, once additional samples are collected, it will be interesting to determine whether any significant relationships exist between vitamin E and the various markers of oxidation that may help answer these questions.

6.4 Summary

Neopterin serves as a marker of immune activation (Hoffmann *et al.*, 2003) and was detected in every sample of pus and carotid plaque analyzed. Pus was associated with a maximum neopterin concentration in the low micromolar range and contained, on average, more pterin than the two atherosclerotic plaque samples. Nevertheless, even the neopterin in one section of plaque was found to approach a micromolar level. It must also be remembered that the plaque research is merely a pilot study and many more samples will be required before strong conclusions, regarding neopterin levels and trends in the atherosclerotic plaque, can be identified.

The lack of correlation between neopterin and various markers of protein and lipid does not support the theory that pterins serve as antioxidants at sites of inflammation. However, it remains possible that such correlations were lost by the time the advanced stage of inflammation was reached and the patient was admitted to hospital for removal of the pus. The positive association between neopterin and the antioxidant, vitamin E, suggests that the antioxidant pterin theory cannot be completely discarded. If any additional correlations did exist, but were rather weak, a sample size larger than 19 would likely be required for statistical significance.

Other relationships in the pus indicated that protein oxidation and lipid oxidation were positively correlated. Furthermore, and as expected for an antioxidant, the increase in vitamin E was associated with a significant decrease in PB-DOPA and TBARS. It should be noted, however, that these relationships were all strongly weighted towards the sample from patient 2. The pus from this patient consistently contained very high levels of oxidized lipid and protein but low quantities of antioxidant.

The pilot study of carotid plaques has confirmed the findings of other research groups. Markers of protein oxidation (PB-DOPA, dityrosine, carbonyls) and lipid oxidation (TBARS) were detected, as was vitamin E. Sample sizes are currently too small to permit the conclusive identification of any relationship between these various parameters. Nevertheless, a preliminary investigation of the plaque from two patients has indicated that antioxidants and products of oxidation are not homogeneous throughout the length of the plaque. Concentrations can vary, and the resulting trend is not necessarily identical between different patients, even when expressed relative to protein or cholesterol rather than plaque weight.

7 General Discussion and Conclusions

A major aim of this thesis was to investigate 7,8-NP's potential antioxidant activity, during both AAPH-induced attack of HMDMs and macrophage-mediated oxidation of LDL. The extent of protein hydroperoxide formation in each model, and 7,8-NP's effect on this process, were specifically studied since most previous research has focussed on lipid rather than protein peroxidation. 7,8-NP was supplied in a single dose, exogenously, as well as continuously from IFN-γ-stimulated macrophages. The existence of neopterin (including oxidized 7,8-NP) was also directly measured for the first time in two inflammatory environments – pus and atherosclerotic plaque.

7.1 The Prevalence of Protein Hydroperoxides

Hydroperoxides were shown to form preferentially on lipid rather than protein in HMDMs exposed to aqueous peroxyl radicals. This is in striking contrast to what has previously been observed during AAPH-induced oxidation of U937 cells (Gieseg *et al.*, 2000a) and red blood cell membranes (Firth, 2001) and irradiation of mouse myeloma cells (Gebicki *et al.*, 2000a; Gebicki *et al.*, 2000b; Du & Gebicki, 2004). Protein was the predominant site of peroxidation in these studies. The culture of HMDMs in 10% human serum is associated with a dramatic accumulation in PUFA-rich lipid (Garner *et al.*, 1997a; Asmis & Jelk, 2000b; Visioli *et al.*, 2000b). Given the susceptibility of PUFAs to oxidative attack (Alexander-North *et al.*, 1994), we therefore hypothesize that the prevalence of lipid hydroperoxides in the HMDM model is due to the high lipid content of these cells. This is of relevance to the atherosclerotic plaque, where macrophages also become lipid-loaded. Of further relevance, break-down products of lipid hydroperoxides have been detected in plaque samples (Suarna *et al.*, 1995; Niu *et al.*, 1999). Peroxyl radical damage should therefore be a significant oxidative stress to macrophages in this environment. As such, lipid hydroperoxide formation in HMDM cells is likely to represent a significant source of the damage to lipid within atherosclerotic plaque. It maybe also be associated with additional adverse consequences for the atherogenic process. For instance, macrophages bearing peroxidized lipids are suggested to enhance LDL oxidation even in the absence of transition metals (Fuhrman *et al.*, 1994).

Lipid hydroperoxide formation and loss of viability were shown to closely correlate, indicating that lipid peroxidation may contribute to the AAPH-mediated death of HMDM cells. The cytotoxicity of lipid peroxidation is also supported by other studies that have noted an increase in cell death upon incubation with lipid hydroperoxides and their aldehyde break-down products (Chisolm *et al.*, 1994; Sandstrom *et al.*, 1995; Colles *et al.*, 1996; Aoshima *et al.*, 1997; Wang *et al.*, 2000; Choudhary *et al.*, 2002). Considering the cytotoxicity of lipid hydroperoxides, much of the peroxidized HMDM lipid could ultimately contribute to growth of the necrotic core, providing yet another means of adversely affecting the atherogenic process.

Exposure of LDL to macrophages in iron(II)-supplemented Hams F10 was consistently shown to result in the formation of 30-40 moles of protein hydroperoxides per mole of LDL. This represents 12-15% of the total LDL hydroperoxides and is proportional to the protein content in LDL. Protein peroxidation was detected at these significant levels whether incubating LDL with the THP-1 macrophage-like cell line or primary HMDM cells. Research arising from this thesis therefore indicates that protein hydroperoxides are a general consequence of all macrophage-mediated LDL oxidation, rather than merely being restricted to one cell type.

Kinetic analysis revealed that macrophage-mediated protein hydroperoxide formation and lipid peroxidation were closely correlated, with the water-soluble 7,8-NP being unable to break this linkage. These findings support the hypothesis that protein hydroperoxide formation on the apoB100 of LDL involves lipid-derived radicals (Gieseg *et al.*, 2003). The close correlation further indicates that, like lipid peroxidation, protein peroxidation can be considered an early product of macrophage-mediated LDL oxidation.

Although protein hydroperoxide formation is a significant and early consequence of macrophage-mediated LDL oxidation, it is not a feature common to all protein-containing substrates. Exposure of BSA to THP-1 macrophages in iron(II)-supplemented Hams F10 did not generate any detectable protein hydroperoxides. It appears that, under these experimental conditions, the lack of lipid associated with a pure protein like BSA limits the peroxidizability of the protein substrate. This result also provides further support for the involvement of lipid-derived radicals during protein hydroperoxide formation on LDL.

Given the importance of macrophages in atherosclerosis and the susceptibility of apoB100 to macrophage-mediated peroxidation, protein hydroperoxides are likely to be generated in significant quantities on LDL in the atherosclerotic plaque. *In vivo* support for this claim arises from the discovery of leucine hydroperoxide and valine hydroperoxide break-down products in atherosclerotic samples (Fu *et al.*, 1998). Just as critically, protein hydroperoxides are known to possess sufficient reactivity to promote additional damage (Gebicki, 1997). Protein hydroperoxide formation may therefore have important implications in atherogenesis.

7.2 The Antioxidant Activity of 7,8-NP

While the exact function of 7,8-NP *in vivo* remains controversial, many studies have provided evidence of an antioxidant activity when this pteridine is incubated at micromolar concentrations *in vitro* (Weiss *et al.*, 1993; Horejsi *et al.*, 1996; Wede *et al.*, 1999; Gieseg *et al.*, 2001a; Gieseg *et al.*, 2001b; Duggan *et al.*, 2002). The current study used two different substrates to further investigate this antioxidant role of 7,8-NP. Both substrates are considered to be of physiological relevance because they represent potential targets of oxidative attack in the inflammatory environments where 7,8-NP synthesis is expected to occur.

In the first study, concentrations of up to 200µM 7,8-NP were unable to inhibit the loss of HMDM viability caused by peroxyl radical attack. This failure to prevent cell death was noted even when the rate of radical flux was reduced by half. Thiol oxidation and lipid hydroperoxide formation were also unaffected by the presence of 7,8-NP. A continuous, endogenous supply of 7,8-NP, produced by IFN-γ-stimulated macrophages, was similarly incapable of protecting HMDMs from AAPH-mediated damage. The lack of effect contrasts with 7,8-NP's inhibition of AAPH-induced damage to BSA (Duggan *et al.*, 2001), erythrocytes (Gieseg *et al.*, 2001a) and the U937 monocyte cell line (Duggan *et al.*, 2002; Baird *et al.*, 2005). Such protection was attributed to 7,8-NP's known ability to efficiently scavenge peroxyl radicals (Oettl *et al.*, 1997). 7,8-NP is soluble in water and was therefore also expected to be a potent scavenger of the water-soluble AAPH peroxyl radicals in the current study. The absence of protection in HMDMs may be caused by 7,8-NP and/or AAPH interacting or partitioning with the cells in such a way that the 7,8-NP is no longer able to successfully scavenge radicals. This interaction/partitioning is likely to arise as a consequence of structural changes that

occur to the HMDMs during differentiation in 10% human serum, ultimately resulting in a lipid-loaded phenotype.

7,8-NP's lack of effect towards HMDMs exposed to AAPH bears more resemblance to the THP-1 monocyte cell line (Baird *et al.*, 2005) than to the U937 monocyte cell line (Duggan *et al.*, 2002; Baird *et al.*, 2005). This provides a cautionary tale against extrapolating directly from cell lines to primary cells. The results also indicate that 7,8-NP should be relatively ineffective at preventing HMDM thiol loss, lipid peroxidation and death caused by the direct attack of external peroxyl radicals. This is particularly relevant in environments like atherosclerotic plaque, where macrophages have become lipid-loaded.

In contrast to the oxidized HMDM results, a micromolar dose of 7,8-NP was able to effectively prevent macrophage-mediated protein hydroperoxide formation on LDL. This protection occurred in a concentration-dependent manner and extended to the entire LDL particle, also preventing lipid peroxidation and vitamin E loss. Inhibition was observed whether the oxidation was mediated by THP-1 macrophages or HMDMs. Further analysis indicated that 7,8-NP did not appear to protect the LDL by modulating macrophage activity but, instead, provided protection by scavenging lipid peroxyl radicals in direct competition to the LDL's vitamin E. Evidence for this theory was obtained from the kinetic data of macrophage-mediated LDL oxidation and 7,8-NP consumption. Cell free LDL oxidation studies have also previously ascribed a lipid radical scavenging mechanism to 7,8-NP (Gieseg *et al.*, 1995; Gieseg *et al.*, 2003).

Oxidative modification of LDL is a key step in the development of atherosclerosis, so 7,8-NP's ability to inhibit this process could have beneficial consequences. Its effectiveness against protein hydroperoxide formation is also of particular interest. Protein hydroperoxides are known to possess sufficient reactivity to cause additional damage (Gebicki, 1997), but direct cellular defences against them appear to be limited, particularly when the protein hydroperoxides are associated with large proteins (Gebicki *et al.*, 2002). 7,8-NP therefore has the potential to provide another line of defence against protein peroxidation and any adverse reactions that may subsequently occur between the reactive protein hydroperoxide moiety and other biomolecules.

Although 7,8-NP was a strong antioxidant when added exogenously in micromolar quantities, the release of nanomolar pterin from IFN-γ-stimulated macrophages yielded contradictory results. Under these conditions, activated THP-1s failed to significantly

prevent LDL oxidation but activated HMDMs dramatically inhibited the oxidation of protein, lipid and vitamin E in LDL. This HMDM effect did not, however, appear to be mediated by 7,8-NP. Medium conditioned by IFN-γ-treated HMDMs retained potent antioxidant activity but contained insufficient pterin to account for the strong degree of protection. Such findings indicate that, although some IFN-γ-activated macrophages can exhibit antioxidant behaviour, 7,8-NP did not play a significant role under the conditions of the current study. The disparity between these two macrophage types also provides another warning against extrapolating directly from cell lines to primary cells.

The contrasting effects of 7,8-NP supplied exogenously, in micromolar amounts, and endogenously, in nanomolar quantities, raises questions regarding its *in vivo* relevance as an antioxidant. Antioxidant activity is clearly dependent on both the concentration of 7,8-NP and the system being investigated.

In vivo, the production of 7,8-NP is expected to occur predominantly at sites of inflammation. As pterin had not previously been quantified at such locations, it was unclear whether the concentrations in these environments would be sufficient to permit any significant antioxidant activity. During this study, neopterin (including oxidized 7,8-NP) was, for the first time, detected in the inflammatory environments of 19 pus and two atherosclerotic plaque samples. An average neopterin concentration of 0.51 ± 0.07μM was determined for the pus, with peak levels even reaching the low micromolar range. Similarly, one section of plaque was found to contain neopterin that approached a micromolar concentration. Although not at the levels required for inhibiting the above macrophage-mediated LDL oxidations, the peak neopterin concentration in pus is comparable to the quantity of 7,8-NP required to delay copper- and THP-1 monocyte-mediated LDL oxidation (Gieseg *et al.*, 1995; Gieseg & Cato, 2003). It also remains possible that, *in vivo*, the rate of oxidation may be slower and/or microenvironments may exist.

As part of a preliminary study, it was premature to investigate the existence of correlations between neopterin and oxidized protein or lipid in the two atherosclerotic plaques. When statistical analysis was applied to the larger pus study, no significant relationships were identifiable between neopterin and PB-DOPA, dityrosine, protein carbonyls or TBARS. While this lack of association does not support the theory that pterins serve as antioxidants at sites of inflammation, it remains possible that such correlations were lost by the advanced stage of inflammation associated with the 19 patients. The positive correlation between neopterin and vitamin E suggests that the

antioxidant pterin theory cannot be completely discarded. Nevertheless, it should be noted that in pus vitamin E is, on average, 45 times more concentrated than neopterin. Both compounds are suggested to scavenge peroxyl radicals at a comparable rate (Oettl *et al.*, 1997). Neopterin would therefore need to act at a different site, or be significantly elevated in a microenvironment, if it were to play any significant antioxidant role compared to vitamin E in the inflammatory surroundings of pus. Neither possibility was able to be investigated in the pus due to the method of isolation and subsequent homogenization of this inflammatory material.

Results derived from this thesis indicate that both the prevalence of protein hydroperoxides, and the ability of 7,8-NP to act as an antioxidant, depend on the system under investigation. Neopterin exists in inflammatory environments but, considering the lack of protection against AAPH-mediated HMDM oxidation and the concentration required to inhibit macrophage-mediated LDL oxidation, strong evidence for an antioxidant activity of 7,8-NP in atherosclerotic plaque is currently lacking.

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Appendix I

Table A.1 Concentration of protein and protein oxidation markers in 19 samples of pus.

Each sample of pus was analyzed for PB-DOPA, dityrosine, carbonyls and total protein. Carbonyl data is not listed for patient 2 because spectrophotometric carbonyl values smaller than the baseline absorbance were consistently recorded in the pus from this patient. Samples were analyzed in triplicate, with results displayed as mean ± SEM.

Patient Number	PB-DOPA (µM)	Dityrosine (µM)	Carbonyls (µM)	Protein (mg/ml)
1	6.14 ± 0.68	4.61 ± 0.84	164.8 ± 47.60	66.46 ± 2.92
2	96.90 ± 4.30	19.28 ± 3.05	/	236.6 ± 7.75
3	11.67 ± 1.50	0.84 ± 0.04	96.90 ± 8.41	35.01 ± 3.31
4	5.38 ± 0.83	0.18 ± 0.02	252.1 ±34.40	26.39 ± 1.81
5	17.83 ± 1.39	0.13 ± 0.02	35.45 ± 4.20	67.91 ± 3.55
6	31.04 ± 2.75	0.09 ± 0.01	52.68 ± 4.20	59.30 ± 1.42
7	4.81 ± 0.43	0.05 ± 0.00	336.4 ± 42.51	41.83 ± 3.54
8	4.16 ± 0.65	0.00 ± 0.00	26.03 ± 6.47	37.91 ± 2.42
9	10.65 ± 1.27	0.57 ± 0.09	5.79 ± 18.79	47.26 ± 1.91
10	7.67 ± 1.15	7.10 ± 0.23	454.9 ± 60.00	46.06 ±2.18
11	2.13 ± 0.13	0.00 ± 0.00	15.00 ± 8.26	20.37 ± 0.22
12	7.22 ± 0.59	0.42 ± 0.02	382.8 ± 9.37	83.39 ± 4.32
13	25.86 ± 0.97	0.09 ± 0.01	94.21 ± 3.63	44.54 ± 2.72
14	19.27 ± 2.56	0.20 ± 0.01	429.2 ± 33.14	66.80 ± 5.72
15	26.43 ± 2.30	0.39 ± 0.04	471.8 ± 14.86	88.85 ± 0.89
16	1.86 ± 0.12	0.29 ± 0.01	164.6 ± 10.17	37.50 ± 0.20
17	2.79 ± 0.14	0.41 ± 0.03	127.0 ± 3.83	51.57 ± 0.95
18	5.33 ± 0.12	0.17 ± 0.02	263.9 ± 8.92	75.47 ± 2.00
19	2.51 ± 0.12	0.12 ± 0.01	180.6 ± 4.80	33.45 ± 1.37

Table A.2 Concentration of TBARS, vitamin E and neopterin in 19 samples of pus.

Each sample of pus was analyzed for TBARS, vitamin E and neopterin (including oxidized 7,8-NP). Samples were analyzed in triplicate, with results displayed as mean ± SEM.

Patient Number	TBARS (μM)	Vitamin E (μM)	Neopterin (μM)
1	0.81 ± 0.10	15.97 ± 1.72	0.69 ± 0.02
2	10.06 ± 1.04	2.96 ± 0.02	0.07 ± 0.00
3	3.84 ± 0.22	7.50 ± 0.60	0.30 ± 0.02
4	1.22 ± 0.18	38.81 ± 1.52	0.40 ± 0.01
5	2.47 ± 0.05	5.37 ± 0.41	0.69 ± 0.02
6	1.01 ± 0.02	14.96 ± 0.23	0.58 ± 0.04
7	4.29 ± 0.06	29.87 ± 0.66	0.65 ± 0.01
8	1.81 ± 0.07	17.66 ± 1.11	0.05 ± 0.00
9	5.98 ± 0.04	18.77 ± 0.92	0.42 ± 0.01
10	1.82 ± 0.00	46.49 ± 2.65	0.52 ± 0.02
11	5.52 ± 0.21	6.32 ± 0.36	0.07 ± 0.00
12	3.40 ± 0.17	35.15 ± 0.67	0.37 ± 0.01
13	5.27 ± 0.11	11.27 ± 0.73	0.89 ± 0.09
14	0.91 ± 0.03	29.35 ± 1.33	0.10 ± 0.00
15	5.28 ± 0.21	29.98 ± 0.69	1.28 ± 0.06
16	2.22 ± 0.00	39.62 ± 0.83	0.64 ± 0.04
17	0.81 ± 0.26	27.99 ± 0.26	0.86 ± 0.07
18	3.33 ± 0.12	21.89 ± 1.49	0.50 ± 0.03
19	2.48 ± 0.08	38.41 ± 0.95	0.54 ± 0.02

Appendix II

The following paper is entitled “Protein Hydroperoxides are a Major Product of Low Density Lipoprotein Oxidation During Copper, Peroxyl Radical and Macrophage-mediated Oxidation”. It includes one figure (Figure 5) that was generated as part of this PhD thesis.

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Protein Hydroperoxides are a Major Product of Low Density Lipoprotein Oxidation During Copper, Peroxyl Radical and Macrophage-mediated Oxidation

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Damage to apoB100 on low density lipoprotein (LDL) has usually been described in terms of lipid aldehyde derivatisation or fragmentation. Using a modified FOX assay, protein hydroperoxides were found to form at relatively high concentrations on apoB100 during copper, 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) generated peroxyl radical and cell-mediated LDL oxidation. Protein hydroperoxide formation was tightly coupled to lipid oxidation during both copper and AAPH-mediated oxidation. The protein hydroperoxide formation was inhibited by lipid soluble α-tocopherol and the water soluble antioxidant, 7,8-dihydroneopterin. Kinetic analysis of the inhibition strongly suggests protein hydroperoxides are formed by a lipid-derived radical generated in the lipid phase of the LDL particle during both copper and AAPH mediated oxidation. Macrophage-like THP-1 cells were found to generate significant protein hydroperoxides during cell-mediated LDL oxidation, suggesting protein hydroperoxides may form *in vivo* within atherosclerotic plaques. In contrast to protein hydroperoxide formation, the oxidation of tyrosine to protein bound 3,4-dihydroxyphenylalanine (PB-DOPA) or dityrosine was found to be a relatively minor reaction. Dityrosine formation was only observed on LDL in the presence of both copper and hydrogen peroxide. The PB-DOPA formation appeared to be independent of lipid peroxidation during copper oxidation but tightly associated during AAPH-mediated LDL oxidation.

Keywords: Low-density-lipoprotein; Protein-hydroperoxide; Protein-bound-DOPA; Free-radical; Lipid-peroxidation; Macrophage; Neopterin

Abbreviations: AAPH, 2,2'azobis(amidinopropane) dihydrochloride; EDTA, Ethylenediaminetetraacetic acid disodium salt; HPLC, high performance liquid chromatography, LDL, low density lipoprotein; PBS, Phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; PB-DOPA, protein-bound-3,4-dihydroxyphenylalanine; TCA, trichloroacetic acid

INTRODUCTION

The oxidation of low density lipoprotein is considered to be a key process in the development and progression of atherosclerosis.[1,2] Oxidised LDL is rapidly taken up by macrophages via the scavenger receptor, leading to the transformation of the macrophages to lipid loaded foam cells.[3] *In vitro*, LDL oxidation begins with the consumption of various endogenous antioxidants and is followed by initiation of the peroxyl radical-mediated chain reaction, leading to peroxidation of polyunsaturated fatty acid esters within the LDL particle.[4] Concomitantly, the structural integrity of the apoB100 protein is lost. Oxidation of the LDL protein moiety results in the loss of select amino acids,[5,6] carbonyl formation,[7] derivatisation with lipid aldehydes,[8] protein fragmentation[5,9] and aggregation through protein cross-linking.[9]

Free radical-mediated protein oxidation also results in the formation of two types of reactive amino acid residues; protein hydroperoxides and protein bound 3,4-dihydroxyphenylalanine (PB-DOPA).[10–13] Both reactive groups are known to form on apoB100 during LDL oxidation[14,15] but, unlike protein carbonyls, protein hydroperoxides and PB-DOPA can initiate further oxidative

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damage to the protein or surrounding biomolecules.[16]

Protein hydroperoxides are formed when a hydroxyl or peroxyl radical removes a hydrogen atom from an amino acid side chain. The resulting carbon-centred radical may then react with oxygen and hydrogen ions to form a hydroperoxide on the amino acid residue.[12] Though relatively stable, protein hydroperoxides readily react with DNA, oxidising nucleotides[17] and forming protein–DNA cross-links.[18] Protein hydroperoxides also oxidise cellular thiols[19] and consume the key cellular antioxidants ascorbate and glutathione.[10] Protein hydroperoxides have been shown to form on various proteins, plasma proteins, lipoproteins and cellular proteins in living cells.[10,15,20,21]

PB-DOPA is formed from tyrosine residues by hydroxyl radical addition to the phenol ring hydrogen atom, followed by hydroxylation of the resulting tyrosyl radical.[11,22] As a reducing agent, PB-DOPA can stimulate further Fenton-mediated hydroxyl radical production by reducing transition metal ions.[23] This mechanism appears to be responsible for PB-DOPA's ability to oxidise DNA.[24] PB-DOPA has been extensively studied using pure proteins, and identified in cataract lens[25] and oxidised lipoproteins during copper-mediated oxidation.[14]

Both PB-DOPA and the breakdown products of protein hydroperoxides have been identified in atherosclerotic plaques[26] and on ApoB in plasma from healthy donors.[27] However, no study has addressed the relative significance of PB-DOPA or protein hydroperoxides during LDL oxidation, nor demonstrated the formation of either protein oxidation product during cell-mediated LDL oxidation. In this study we show that protein hydroperoxides are a major product of copper ion, peroxyl radical and cell-mediated LDL oxidation. The lipid soluble antioxidant, α-tocopherol (vitamin E), and the water soluble antioxidant, 7,8-dihydroneopterin, have been used to examine the relationship between lipid oxidation and protein hydroperoxide formation during LDL oxidation. 7,8-dihydroneopterin is a macrophage-synthesised antioxidant, which has previously been shown to inhibit lipid oxidation on LDL and protein hydroperoxide formation on albumin exposed to peroxyl or hydroxyl radicals.[28,29]

MATERIALS AND METHODS

All chemicals and reagents were of AR grade or better and obtained from the Sigma Chemical Company (USA) or BDH Chemicals New Zealand Limited. 7,8-dihydroneopterin was obtained from Schirck's Laboratories, Switzerland. 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) was supplied by Aldrich Chemical Company Inc. Ethylenediaminetetraacetic acid disodium salt (EDTA) was supplied by BDH chemicals.

All solutions were prepared with high purity water from a NANOpure ultrapure water system from Barnstead/Thermolyne (Iowa, USA). Phosphate buffered saline (PBS) (160 mM sodium chloride, 10 mM sodium phosphate buffer pH 7.4), was treated with chelex-100 resin (BioRad, Richmond, USA) to remove trace amounts of redox active metal ions.

EDTA-plasma was prepared from blood taken by venipuncture from healthy female and male donors (age 20–45 years). The EDTA-plasma from groups of five donors was pooled and frozen at −80°C in 0.6% sucrose for up to 3 months.[30] LDL was prepared by a single 22 h ultracentrifugation of the pooled EDTA-plasma using a buoyant density four step discontinuous gradient in a Beckman SW41 rotor.[31] For oxidation experiments, the LDL was desalted by 24 h of dialysis with four changes of nitrogen-gassed PBS containing 0.5 g/l of chelex-100 resin. The LDL molar concentration was determined by enzymatic cholesterol determination using the "Chol MPR 2" kit supplied by Roche Chemicals (New Zealand), assuming an LDL molecular weight of 2.5 MDa and a cholesterol content of 31.6%.[30]

The α-tocopherol concentration in LDL was increased by incubating 4 ml of plasma with 40 μl of 100 mM α-tocopherol in ethanol under argon gas for 4 h at 37°C with gentle shaking.[32] The LDL was then purified as described above.

LDL (100 nM) in PBS was oxidised in a glass flask in the dark at 37°C in a Bioline shaker (Edwards Instruments Co. Australia), which gently swirled the flask at 80 rpm.

Protein hydroperoxides were isolated by acid precipitation and solvent lipid extraction, before analysis using a modified FOX assay.[21,33,34] The assay was performed in triplicate, by adding 140 μl of 72% w/v trichloroacetic acid (TCA) to 1 ml samples of LDL (100 nM) and incubating on ice for 10 min. The protein was pelleted by a 5 min centrifugation at 23,000*g*, 4°C. The pellet was washed by vortexing in 1 ml of 1:1 methanol/chloroform before centrifugation. The resulting pellet was allowed to dry in air at room temperature for 5 min, before being suspended in 900 μl of ice cold 50% v/v glacial acetic acid. Fifty microliter each of 5 mM ferrous ammonium sulphate and xylenol orange in 25 mM H_2SO_4 was added to the protein suspension. The mixture was incubated at room temperature for 30 min, during which time ferrous ions were oxidised to ferric ions by the hydroperoxides. Ferric ions are detected by the formation of a coloured complex with xylenol orange, which is measured by absorbance at 560 nm against a water blank. An extinction coefficient of 48,000 M cm was

used to calculate the concentration of protein hydroperoxide.[33]

For cell-mediated oxidation experiments, 3 ml of the LDL-containing media was centrifuged at 400*g* to eliminate any cellular material. The supernatant was then incubated with 420 μl of 72% w/v TCA on ice for 10 min before centrifugation at 4100*g* for 15 min at 4°C in thick walled glass centrifuge tubes. The pellet was washed with chloroform/methanol and assayed for protein hydroperoxides as described above.

Lipid oxidation was monitored by removing aliquots of the reaction mixture and measuring the formation of conjugated dienes by the increase in absorbance at 234 nm.[35] As the breakdown product of AAPH absorbs at 234 nm, the absorbance of an incubated AAPH solution was subtracted from the LDL oxidation mixture.

PB-DOPA and dityrosine were measured by acid hydrolysis and HPLC analysis, as previously described.[11,36] The samples were prepared in triplicate by placing 900 μl of LDL solution into glass "Durham" tubes, 50 × 7.5 mm (Samco, USA), containing 10 μl of 20 mg/ml butylated hydroxytoluene (BHT) in methanol, 10 μl of 100 mg/ml EDTA and 140 μl of 72% TCA. Samples were incubated on ice for 10 min before centrifugation at 10,000*g* for 15 min at 4°C. The supernatant was removed and the pellet washed by vortexing with 500 μl of 20°C acetone, then 500 μl of diethyl ether, with centrifugation between each wash to pellet the protein. After drying in air, the sample tubes were placed in a "picotag hydrolysis vial" (Waters, USA) and subjected to gas phase acid hydrolysis over 16 h at 110°C.[11] Concentration of DOPA and dityrosine in the hydrolysate was determined by reverse phase HPLC, with fluorescence detection as previously described.[36,37] The dityrosine standard was prepared by oxidation of tyrosine with horseradish peroxidase and purification on DEAE-sephacel (Sigma).[38] The concentration of the purified dityrosine was determined by measuring the absorbance at 315 nm, using an extinction coefficient of $E_{315} = 5080\,M$.[39]

Suspension cultures of THP-1 cells were maintained in RPMI 1640 media, supplemented with 5% heat inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified incubator with 5% CO_2 at 37°C. The cells were transformed to adherent macrophage-like THP-1 cells, by treatment for 7 days with 100 ng/ml phorbol 12-myristate 13-acetate (PMA), to give a cell density of 1×10^5 cells/ml in a standard 6 well plate.[40] Oxidation experiments were conducted in triplicate, using Hams F10 media, supplemented with 3 μM $FeSO_4$, after first washing the cells three times in warm PBS. Filter sterilized LDL was added to the media to give a final concentration of 40 nM before incubation for various times at 37°C in 5% CO_2.

The results shown are from single experiments, representative of a minimum of three separate experiments. The data points shown in the figures are the means and standard errors of triplicate analysis taken from a single flask.

RESULTS

The oxidation of 100 nM LDL by 5 μM copper ions at 37°C over 2 h resulted in the formation of 311 ± 20 moles of total hydroperoxides/mole of LDL using the standard protein FOX assay with acid precipitation[33] (data not shown). Removing lipid from the sample by acid precipitation and solvent washing, as described in the methods, reduced the FOX-measured hydroperoxides to 52 protein hydroperoxides per LDL particle. This equates to 16% of the combined protein and lipid hydroperoxide value. Unoxidised LDL gave a background reading of 0.01 absorbance units, which corresponds to a reading of 2 mole per mole LDL. Analysis of delipidated and native unoxidised LDL showed the delipidation procedure did not generate compounds which reacted in the FOX assay. Treatment of the oxidised LDL with sodium borohydride removed all of the FOX-assayed reactivity generated by the copper oxidation. The susceptibility to sodium borohydride, and the effect of acid and solvent washing, confirm the presence and formation of significantly large levels of protein hydroperoxides during LDL oxidation.[21,41] The change from sulphuric acid[33] to 50% glacial acetic acid to partially solubilise the precipitated apoB100 protein is a critical modification of the FOX assay, which enabled LDL protein hydroperoxides to be measured. It was also found to be important to keep the glacial acetic acid on ice before conducting the FOX assay at room temperature.

Kinetic analysis of the oxidation showed that protein hydroperoxide formation closely paralleled the formation of conjugated dienes with both copper and AAPH-mediated oxidation (Figs. 1 and 2). No protein or lipid oxidation was observed in the oxidant-free controls over the same time period. Like the conjugated dienes formed during lipid peroxidation, the protein hydroperoxide kinetics show a lag period followed by a rapid formation phase. In lipid peroxidation this rapid oxidation phase is usually termed the propagation phase as it is during this part of the reaction that a lipid radical chain reaction forms.[35] The maximum protein hydroperoxide formation rate with copper ions was calculated at 1 nM/s while the lipid conjugated diene formation was calculated at 4.3 nM/s. Following the copper-mediated propagation phase, both protein and lipid oxidation reached a plateau (Fig. 1A). With AAPH oxidation, the maximum protein hydroperoxide and

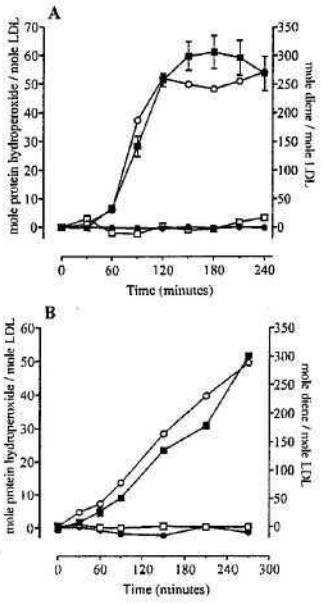

FIGURE 1 Formation of protein hydroperoxides on LDL. LDL (100 nM) in PBS was oxidised by either (A) 5 μM Cu^{++} or (B) 1 mM AAPH at 37°C. Protein hydroperoxides were measured in triplicate by the modified FOX assay and displayed as mean ± standard error. Conjugated dienes were determined by measuring the absorbance at 234 nm. The data displayed shows (●) protein hydroperoxide control; (■) protein hydroperoxide with oxidant, (□) conjugated diene control, (□) conjugated diene with oxidant.

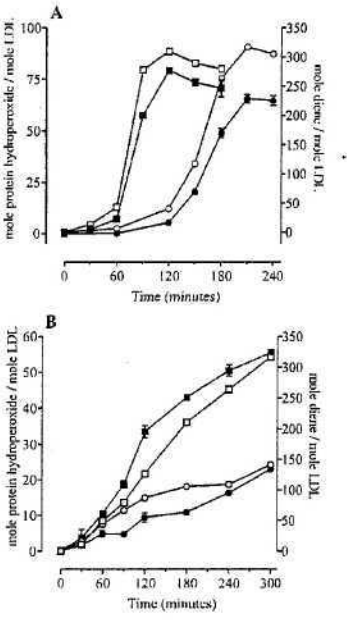

FIGURE 2 Effect of α-tocopherol loading on protein hydroperoxide formation on LDL. (A) Native LDL (100 nM with 8.5 α-tocopherols per LDL particle) and α-tocopherol-loaded LDL (100 nM with 54 α-tocpherols per LDL particle) was incubated with 5 μM copper at 37°C. (B) Native LDL (100 nM with 8 α-tocopherols per LDL particle) and α-tocopherol-loaded LDL (100 nM with 70 α-tocopherols per LDL particle) was incubated with 2 mM AAPH at 37°C. Samples were removed at selected time points for protein hydroperoxide analysis by the modified FOX assay and lipid oxidation by measurement of the conjugated diene absorbance at 234 nm. Each protein hydroperoxide value is the mean ± standard error of three replicates. Blank controls and the zero time point were subtracted to show the change in oxidation products over time. The data displayed shows (■) native LDL protein hydroperoxides; (●) α-tocopherol-loaded LDL protein hydroperoxides; (□) native LDL dienes; (○) α-tocopherol-loaded LDL dienes.

diene formation rates were 0.3 and 2 nM/s, respectively (Fig. 1B).

To determine the possible linkage between the oxidation of LDL's protein and lipid components, the effect of a lipid soluble and a water soluble antioxidant on the oxidation process was investigated. Increasing the α-tocopherol content of LDL, from 8.5 to 54 α-tocopherol per LDL particle, doubled the length of the lag phase during copper-mediated LDL oxidation (Fig. 2A). The additional α-tocopherol had no significant effect on the propagation rates of lipid and protein oxidation and did not disrupt the linkage observed between protein hydroperoxide and diene formation. During AAPH-mediated oxidation, the increase in α-tocopherol caused a dramatic reduction in the concentration of both dienes and protein hydroperoxides. This inhibition was particularly apparent for the protein hydroperoxides from the 30 min time point onwards (Fig. 2B).

Like α-tocopherol, the presence of the water soluble antioxidant 7,8-dihydroneopterin lengthened the lag phase but had little impact on the propagation phase kinetics of dienes and protein hydroperoxides during copper-mediated oxidation (Fig. 3A). In the particular example shown, diene values did peak much earlier than the hydroperoxides during oxidation of the native LDL but this was not consistently observed. During AAPH oxidation, the presence of 7,8-dihydroneopterin caused a lengthening of the lag phase (Fig. 3B). At first glance, lipid peroxidation appears the most affected by the 7,8-dihydroneopterin in the propagation phase but close inspection of the data shows that the presence of 7,8-dihydroneopterin virtually blocked the formation of dienes during the lag phase. In the absence of 7,8-dihydroneopterin, 40 dienes per

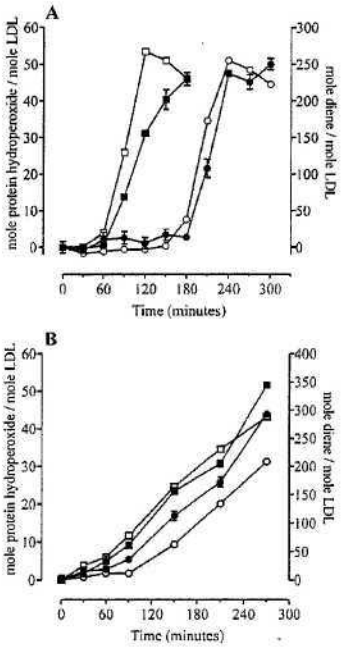

FIGURE 3 Effect of water soluble 7,8-dihydroneopterin on protein hydroperoxide formation in LDL. LDL (100 nM) in PBS was oxidised by either (A) 5 μM Cu^{++} or (B) 1 mM AAPH at 37°C in the presence or absence of 2 μM 7,8-dihydroneopterin. Samples were removed at selected time points for protein hydroperoxide analysis by the modified FOX assay and lipid oxidation by measurement of the conjugated diene absorbance at 234 nm. Each protein hydroperoxide value is the mean ± standard error of three replicates. Blank controls and the zero time point were subtracted to show the change in oxidation products over time. The data displayed shows (■) native LDL protein hydroperoxides; (●) 7,8-dihydroneopterin + LDL protein hydroperoxides; (□) native LDL dienes; (○) 7,8-dihydroneopterin + LDL dienes.

LDL particle had formed at the end of the lag phase (60 min time point) compared to only 12 dienes per LDL particle at the end of the lag phase in the presence of 7,8-dihydroneopterin. Protein hydroperoxide formation was also reduced by 7,8-dihydroneopterin during this lag period.

PB-DOPA and dityrosine formation were also observed during LDL oxidation (Table I). Only PB-DOPA was observed after a 3 h LDL oxidation with copper alone, while the addition of hydrogen peroxide resulted in both the detection of dityrosine and a doubling of PB-DOPA. Hydrogen peroxide alone was unable to cause the formation of either tyrosine oxidation product.

The kinetics of PB-DOPA formation during copper oxidation were significantly different to the rate of

TABLE I Fenton formation of PB-DOPA and PB-Dityrosine on ApoB100

Treatment	DOPA mmoles/ mole LDL	Dityrosine mmoles/ mole LDL
LDL	0	0
LDL + H_2O_2	0	0
LDL + Cu^{+2}	157 ± 20	0
LDL + Cu^{+2} + H_2O_2	348 ± 1	70 ± 0.1

LDL (100 nM) was incubated in PBS in the presence and absence of 5 μM copper chloride or 5 mM hydrogen peroxide at 37°C for 3 h. The zero time point was subtracted from the value at 3 h of each treatment as there is a small but significant background level of both oxidation products in LDL purified from human plasma. The data shown is the mean ± standard error of three replicates.

diene formation (Fig. 4A). The PB-DOPA continued to increase well after the peak in diene formation. In contrast, PB-DOPA formation appeared tightly coupled to the lipid diene formation during AAPH-mediated oxidation (Fig. 4B). AAPH-mediated

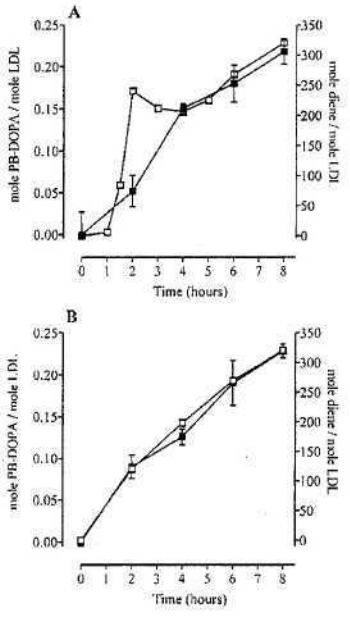

FIGURE 4 Formation of PB-DOPA during copper and AAPH-mediated LDL oxidation. LDL (100 nM) was incubated in PBS at 37°C, with and without either (A) 5 μM copper ions or (B) 2 mM AAPH. Triplicate samples were removed at the indicated time points for protein extraction, acid hydrolysis and HPLC analysis for DOPA. Lipid oxidation was monitored by measuring the absorbance of the conjugated dienes at 234 nm. No increase in PB-DOPA or dienes was observed in the oxidant-free controls. Blank controls and the zero time point were subtracted to show the change in oxidation products over time. Each PB-DOPA value is the mean ± standard error of three replicates. The data displayed shows (■) PB-DOPA; (□) dienes.

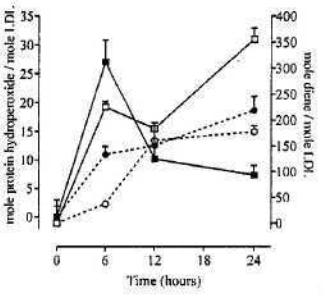

FIGURE 5 LDL (40 nM) was incubated with and without adherent PMA treated THP-1 cells in iron supplemented Hams F10 media at 37°C. At the indicated time points, wells were sampled and analysed for protein hydroperoxides by the modified FOX assay, and lipid dienes by the absorbance at 234 nm. Each data point is mean ± standard error of three wells. Blank controls and the zero time point were subtracted to show the change in oxidation products over time. Data displayed shows protein hydroperoxide formation in the presence (■) and (●) absences of cells. Conjugated dienes are shown in the presence (□) and (○) absences of cells.

DOPA formation on LDL was unexpected because hydroxyl but not peroxyl radicals were believed to generate PB-DOPA.[10,42] A Fenton-type mechanism, using hydrogen peroxide generated from AAPH, was unlikely because the addition of catalase failed to inhibit PB-DOPA formation. Unlike PB-DOPA, no dityrosine was generated during either the copper or AAPH-mediated LDL oxidation. Control experiments confirmed that, in the absence of copper or AAPH, no significant PB-DOPA or dienes were detectable (data not shown).

In comparison to the protein hydroperoxide, PB-DOPA and dityrosine were relatively minor reaction products. Only one DOPA was detected per 380 protein hydroperoxides (comparing data in Fig. 1 with Table I) and we therefore did not examine the reaction kinetics of tyrosine oxidation further.

Incubation of LDL with macrophage-like THP-1 cells resulted in the oxidation of both protein and lipid (Fig. 5). Protein hydroperoxides and dienes peaked at 6 h, at concentrations of 27 moles per mole of LDL and 225 moles per mole of LDL, respectively. A second rise of 234 nm absorbing material was observed at 24 h, though this is most likely due to advanced lipid oxidation products which also absorb at 234 nm.[35] Protein hydroperoxide and diene formation both occurred at slower rates in the absence of cells due to auto-oxidation in the presence of iron and air.

DISCUSSION

It has long been recognised that damage to the apoB100 moiety of LDL is an important consequence of LDL oxidation.[5] In addition to the more classical types of damage to LDL, we have now clearly shown that the formation of protein hydroperoxides on the LDL apoB100 protein is a significant form of LDL modification. The maximum amount of protein hydroperoxide recorded during copper oxidation was 79 protein hydroperoxides per LDL particle, compared with 309 conjugated dienes per particle (Fig. 2). This protein hydroperoxide concentration is six times greater than the reported level of protein carbonyls formed during copper-mediated LDL oxidation.[7]

Modifying the sample preparation procedure to include protein precipitation and solvent lipid extraction enabled the FOX assay to specifically measure protein hydroperoxides. Degradation of the FOX-measured protein hydroperoxides by reduction with sodium borohydride[43] confirmed early work, which detected protein hydroperoxide in oxidised LDL using an iodometric assay.[15]

Protein hydroperoxide and conjugated diene formation paralleled each other during both copper and AAPH-mediated LDL oxidation (Fig. 1). Copper mediated LDL oxidation appears to involve a Fenton-like process occurring at specific copper binding sites on the surface of the LDL particle, possibly on the apoB100.[30,44] In contrast, AAPH is a water soluble peroxyl radical generator, capable of forming protein hydroperoxides in pure protein solutions[21] and oxidising LDL via the formation of lipid peroxyl radicals.[45] Despite the differences in these two radical generating systems, a high level of similarity was observed in the kinetics of protein hydroperoxide and diene formation. This suggests that the oxidation of both moieties was initiated by a common radical intermediate.

The inability of α-tocopherol and 7,8-NP to uncouple the oxidation of the lipid and protein components of LDL during copper and AAPH-mediated oxidation suggests that radical intermediates of the lipid peroxidation are responsible for protein hydroperoxide formation in LDL (Fig. 2). α-Tocopherol's location within the lipid component of LDL supports a lipid origin for the radicals responsible for initiating protein hydroperoxide formation. 7,8-dihydroneopterin and α-tocopherol inhibit LDL oxidation by competing for the lipid peroxyl radical during both copper and AAPH-mediated LDL oxidation.[28] Lipid alkoxyl radicals are unlikely to be involved in protein hydroperoxide formation as these radicals predominate in the latter stages of lipid oxidation, after protein hydroperoxide formation has peaked. The most likely lipid radical responsible for protein hydroperoxide formation on LDL is therefore the chain-propagating lipid peroxyl radical. The finding that α-tocopherol can prevent protein hydroperoxide formation on apoB100 (Fig. 2), potentially broadens its role to protection of the entire LDL particle from oxidative insult.

The lipid soluble antioxidant probucol has also been shown to protect apoB100 by inhibiting lipid peroxidation, carbonyl formation and protein fragmentation.[7,46] It is possible that a significant part of this apoB100 damage results from the breakdown of protein hydroperoxides. Degradation of hydroperoxide on the α-carbon of model peptides has been shown to cause fragmentation,[47] while the decomposition of hydroperoxide on the amino acid residue side chain generates carbonyl groups.[48] The amino acids lysine, histidine and proline have been identified as highly labile in apoB100 during copper-mediated LDL oxidation.[5] This loss has previously been attributed to protein fragmentation or carbonyl formation. These three amino acids are now known to also readily form amino acid hydroperoxides during exposure to hydroxyl radicals.[43] This suggests that some of the previously described amino acid loss during LDL oxidation is probably due to protein hydroperoxide formation. It is interesting to note that α-tocopherol has also been shown to inhibit protein fragmentation of apoB100 during copper oxidation.[46]

It is generally assumed that, because AAPH is water soluble, AAPH-derived peroxyl radicals will form in the aqueous phase of a reaction mixture. As a result, LDL protein hydroperoxides would be expected to form via direct attack by AAPH peroxyl radicals in the aqueous phase, as is observed with albumin.[29] The lipid soluble α-tocopherol should have had little or no affect on the LDL protein hydroperoxide formation during our experiments. Though water soluble, 95% of AAPH can partition in the lipid phase of SDS-stabilized linoleic acid micelles.[49] This suggests the majority of the AAPH peroxyl radicals in our experiments are forming in the lipid fraction of the LDL particle, not the aqueous phase. If the AAPH derived radicals were forming in the aqueous phase, they would be able to directly attack the ApoB 100 to form protein hydroperoxide, with out α-tocopherol having any affect. This model of AAPH-mediated oxidation is further supported by the finding that the length of the LDL lag phase is directly proportional to the concentration of α-tocopherol, but only during AAPH-mediated oxidation.[50]

Macrophage cells are considered to play a key part in the modification of LDL within the arterial wall. The novel finding that cell-mediated LDL oxidation also generates significant amounts of protein hydroperoxides suggests that protein hydroperoxides could form in atherosclerotic plaques (Fig. 5). Analysis of atherosclerotic plaque has shown the presence of hydroxyleucine and hydroxyvaline, known breakdown products of protein hydroperoxides.[26] Considering the known reactivity of protein hydroperoxides with thiols and ascorbate,[10] it is possible that they play a role in plaque formation.

In comparison to protein hydroperoxide formation, oxidative modification of tyrosine does not appear to constitute a major reaction during the oxidation of LDL. The concentration observed during copper-mediated oxidation after 8 h was only one PB-DOPA per five LDL particles (Fig. 4A). PB-DOPA concentrations have been reported as high as eight DOPA per LDL particle, but this was only after 8 days of oxidation.[14] Measurement of amino acid loss during copper-mediated LDL oxidation also confirmed tyrosine to be a minor reaction target during copper-mediated LDL oxidation.[5]

The appearance of PB-DOPA during AAPH-mediated LDL oxidation was unexpected (Fig. 4B), as previous studies had shown that only hydroxyl radicals were capable of initiating DOPA formation from tyrosine.[10] The strong coupling observed between the diene and DOPA formation strongly suggests that the lipid peroxyl radical is involved in the mechanism. Spin trap studies have shown peroxyl and alkoxyl radicals can undergo a range of fragmentation reactions releasing superoxide radicals, which can dismutate to hydrogen peroxide.[51] The lack of effect by catalase suggests that either there is another mechanism leading to DOPA formation or the reaction site where the hydrogen peroxide is reacting to form hydroxyl radicals is not accessable to the catalase enzyme.

In contrast, during copper-mediated LDL oxidation, the PB-DOPA formation appeared to be relatively independent of the diene formation (Fig. 4A). A direct copper/tyrosine interaction rather than a lipid radical intermediate appears to be involved, though this has not been observed in our studies with albumin and copper.[42]

Significant dityrosine formation was only observed under the most oxidative of conditions, when hydrogen peroxide was added to the copper/LDL reaction mixture. After 3 h of incubation only one dityrosine per 14 LDL particles was detected (Table I). This may reflect the requirement of two tyrosyl radicals to occur in close proximity to each other to form dityrosine.[52] Studies on lens proteins have also found that hydrogen peroxide has to be present with the copper ions for dityrosine to be generated by the Fenton reaction.[53]

This study clearly demonstrates that protein hydroperoxides are a significant product of LDL oxidation under a variety of oxidation systems, including cell-mediated oxidation. Others have clearly shown that protein hydroperoxides are found in atherosclerotic plaques[26] and will react to consume thiols and ascorbate.[12] This raises the possibility that protein hydroperoxides may

contribute to some of the pathological behaviour of oxidised LDL *in vivo*.

Acknowledgements

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